Retinoic Acid Biosynthesis by Normal Human Breast Epithelium Is via Aldehyde Dehydrogenase 6, Absent in MCF-7 Cells

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ABSTRACT

Retinoic acid (RA) is the form of vitamin A that controls differentiation and proliferation of epithelia. Our previous work established that normal breast epithelia synthesize RA from retinol, an ability retained by three immortalized but nontumorigenic cell lines but lost in five of six breast cell lines. In this work, we characterize the cause of this defect in one of the lines, the MCF-7 line. We have determined that the immortalized but nontumorigenic cell line, MTSV1.7, capable of synthesizing RA from both retinol and retinal, contains a retinaldehyde dehydrogenase activity for the second step in RA biosynthesis. We have identified it, after isolation, as a previously described enzyme, aldehyde dehydrogenase 6 (ALDH6). Immunohistochemical analysis of normal human breast with antibodies to ALDH6 showed expression of this enzyme in the glandular epithelia colocalized with cellular RA-binding protein type II, a possible marker for certain cells able to synthesize RA. ALDH6 was not present in MCF-7 cells, and these cells were unable to oxidize retinal to RA in culture. When MCF-7 cells were then transfected with ALDH6, they regained the ability to oxidize retinal to RA as well as some ability to synthesize RA when provided with retinol. This suggests that loss of ALDH6 expression is the defect in RA biosynthesis in these cells. Identification of ALDH6 as the retinaldehyde dehydrogenase present in normal human breast epithelium provides the first tool necessary for studying the loss of RA synthetic ability in cancer cells and the relationship of this process to malignant transformation.

INTRODUCTION

An important biological activity of vitamin A is the control of the differentiation and proliferation of various epithelia of the body. The studies of Wollbach and Howe (1) in 1925 demonstrated that the absence of vitamin A leads to the uncontrolled proliferation of epithelial stem cells that fail to differentiate to the normal phenotype in many lining epithelia. Histological changes in vitamin A-deficient rats showed both the abnormal differentiation of epithelia to a squamous, keratinizing phenotype and the hyperproliferation of this abnormal epithelium (1). Interestingly, the histological changes they observed at some sites included invasion of other cellular layers by the proliferating cells with incorporation of new vasculature, leading them to observe that this was “growth power of neoplastic potentiality.”

The ability of vitamin A acid, RA, to prevent or reverse such changes (2, 3) has prompted numerous investigations of the possible involvement of members of the vitamin A family, the retinoids, in the development or prevention of certain cancers. Some of these efforts have been directly directed toward identifying changes in the cellular components important for retinoid action, beginning 25 years ago (4).

Materials and Methods

Cell Culture. MTSV1.7 cells were a gracious gift of Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom) and were cultured as recommended in DMEM/F12 50:50 mix supplemented with 10% FBS and 0.5 μg/ml hydrocortisone. MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM/F12 50:50 mix, 10% FBS, and 0.5 μg/ml insulin. NMU cells from American Type Culture Collection, derived from a rat mammary adenocarcinoma, were cultured in DMEM/F12 50:50 mix with 10% FBS.

To measure RA synthesis in culture, cells were seeded in 60-mm dishes and grown until just confluent. Normal medium (described above) was removed and replaced with 3 ml of medium containing 10 μM BSA and 2 μM retinol or retinal (provided in DMSO). Cells were incubated with retinoids for 4 h, then the cells were scrapped into the medium, and cells and medium were extracted together. For quantitation, a duplicate plate of cells was trypsinized, pelleted, and lysed by sonication, and the protein amount was determined by BCA Assay kit (Pierce, Rockford, IL).

Transient transfection of human ALDH6 into MCF-7 cells was performed using SuperFect from Qiagen (Valencia, CA), following the manufacturer’s protocol with slight modification. Cells were seeded at ~50% confluence in 60-mm dishes, and 5 μg of DNA were complexed for 10 min at room temperature with 30 μl of SuperFect in 150 μl of serum-free medium. One ml
of normal medium was added to the complex and applied to cells for 3 h, after which complexes were left on cells and 3 additional ml of normal medium were added to the cells. Cells were assayed 36 h after transfection. Cells were transfected with the human ALDH6 sequence cloned into pcDNA 3.1(−) (Invitrogen, Carlsbad, CA) or vector alone as control.

**RT-PCR of RALDHs.** To investigate possible expression of known RALDHs, RT-PCR was performed with oligo(dT)-primed cDNA from MTSV1.7 cell RNA, using a general forward primer designed to a highly conserved region of the ALDH family, 5′-TTTGCAGATGCCTACCATGGGAC-3′, or specific forward primers for RALDH1 and 2, 5′-TGAAGGCAGGGAAGAA-3′ and 5′-CTTGGATCCCATCTAAGGAG-3′, respectively. Forward primers were used in conjunction with a common reverse primer, 5′-CCATTCCAGAGACTTGGAG-3′.

**Isolation and Identification of ALDH6 in MTSV1.7 Cells.** MTSV1.7 cells were grown in 150-mm culture dishes, trypsinized, pelleted, and stored at −70°C before use. Cells from 20 150-mm plates were harvested, and the pellets were resuspended in 16 ml of buffer A [0.02 M Tris acetate (pH 8.3), 5 mM DTT] and sonicated three times for 30 s. After centrifugation at 15,000 × g for 20 min in a Beckman Optima TL Ultracentrifuge, the supernatant was applied to an HPLC ion-exchange column (LKB TSK DEAE-5PW column, 8 mm × 7.5 cm) equilibrated in buffer A at 1 ml/min. After one column volume of wash with buffer A, a gradient elution was started from buffer A to 500 mM NaCl containing 10% glycerol (pH 7.5). The 100,000 × g supernatant was incubated with 4 ml of a 50% slurry of nickel-nitro-triacetic acid resin (Qiagen) and then loaded into a column for washing with wash buffer [40 mM imidazole acetate, 1 mM n-octyl glycoside, and 10% glycerol (pH 8.0)]. The recombinant protein was eluted from the column with 200 mM imidazole acetate, 2 mM βME, 250 mM NaCl, 1 mM n-octyl glycoside, and 10% glycerol (pH 8.0). Purity of recombinant protein was assessed by visualization of only a single band by Coomassie Blue staining.

**Antibodies, Western Blots, and Immunohistochemistry.** A peptide sequence of ALDH6 with significant difference from RALDH-1 and RALDH-2 was chosen for immunization. The peptide, CCGSAGEDKGLFPK, was synthesized by Peptidogenics (Livermore, CA). Two mg were conjugated to keyhole limpet hemocyanin with the Pierce InJet Maleimide Activated Kit, and two rabbits were immunized intradermally with Hunter’s TiterMax Gold (CytRx, Norcross, GA) as adjuvant. After 5 weeks, rabbits were boosted intramuscularly, and after 10 days serum was obtained. The IgG fraction was purified from serum by incubation of Office A Protein A column (Pierce), and antibodies to ALDH6 were purified by affinity chromatography with the immunizing peptide immobilized on a Sulfolink column (Pierce).

**Analysis of ALDH6 by Western blot was performed by separation of cytosol or recombinant protein samples (ALDH6 produced as described, or rat RALDH-2, a gift of Marcia Newcomer, Baton Rouge, LA) on 10% SDS-PAGE and electrophoretic transfer to polyvinylidene difluoride membrane. After blocking in 5% milk, blots were incubated with affinity-purified anti-serum at 1:200 dilution overnight at 4°C. Immunodetection was performed with horseradish peroxidase-conjugated secondary antibody against rabbit IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at 1/5000 dilution for 1 h, followed by detection in enhanced chemiluminescence reagents.**

**RESULTS**

**Synthesis of RA from Retinal Is Impaired in MCF-7 Cells.** We reported previously that the MCF-7 cell line was incapable of synthesis of RA when provided with retinol. The synthesis is a two-step process, with the first step being the oxidation of retinol to retinal, followed by oxidation of retinal to RA. MTSV1.7 cells, an immortalized but nontumorigenic breast epithelial cell line shown previously to be able to synthesize RA in culture when provided with retinol, were, as expected, also able to convert retinal to RA (Fig. 1). However, incubation of MCF-7 cells with retinal gave almost no detectable production of RA. MTSV1.7 cells produced an average of 370 ± 140
pmol RA (average ± SD; n = 7) per confluent 60-mm dish of cells, compared with only 10.2 ± 0.5 pmol RA (n = 3) recovered from comparable cultures of MCF-7 cells. This indicates that at least part of the defect in the ability of MCF-7 cells to synthesize RA from retinol occurred at the second step of RA biosynthesis. Interestingly, mRNA for either of two known RALDHs, RALDH-1 and RALDH-2 (17–20), was not detected by RT-PCR in the MTSV1.7 cell line with primers able to detect specifically either rat or human mRNA. However, a band was observed with degenerate primers designed to detect any member of this family of ALDHs (data not shown). Sequencing of this product revealed the presence of mRNA for family member ALDH6 (14), which is proposed to be a RALDH but has not yet been confirmed at the time of this result. Nucleotide identity of the 565-bp RT-PCR product, excluding the primer sequences, and the ALDH6 sequence (GenBank accession no. HSU07919) was 100%.

Identification of the RALDH in MTSV1.7 Cells as ALDH6. To determine whether ALDH6 was indeed the enzyme responsible for the cellular conversion of retinal to RA, cytosol was collected from MTSV1.7 cells and assayed, revealing potent RALDH activity. The activity was assayed in a two-column purification procedure, migrating as a single component during the first separation on an anion-exchange HPLC column (data not shown). Fractions containing the activity were pooled and applied to a HPLC size-exclusion column. A single peak of activity was again observed, eluting at a position consistent with a molecular weight of $M_r \sim 100,000,000$ (Fig. 2A). The fractions containing activity (27–33) were then examined by SDS-PAGE. A number of bands were visible after protein staining, but only one, with an apparent molecular weight of $M_r 56,000$, corresponded in intensity as judged by visual inspection with the measured activity in each fraction (Fig. 2B). Members of this ALDH...
family are normally either dimers or tetramers. Under the conditions used here, the enzyme appeared to be a dimer, consistent with the shift in apparent molecular weight between the sizing column elution position and migration position after SDS-PAGE.

This band was excised and subjected to tryptic digestion, and the mass of the principal peptide products was determined by mass spectroscopy. This pattern of fragments was matched against the predicted masses of theoretical tryptic digests of known proteins. The band was identified as ALDH6 by this procedure because all but two of the observed peaks in the mass range examined corresponded (within 1 mass unit) to a predicted tryptic peptide from the ALDH6 sequence (Fig. 3). The murine homologue of ALDH6 (also termed RALDH-3) has now been cloned and is believed to be one of the RALDHs functioning in the embryo (21–23), consistent with our results here.

Western Blot Analysis and Immunohistochemical Localization of ALDH6. We produced polyclonal antibodies in rabbit against a peptide sequence from ALDH6 that was distinct from other known members of this family. Western blot analysis of cytosol from MTSV1.7 cells with the affinity-purified IgG population from the antiserum showed a single band of the appropriate size but no band for cytosol from MCF-7 cells (Fig. 4). The antibody preparation did not react with recombinant rat RALDH-2.

Expression of ALDH6 in normal human breast epithelium was demonstrated by immunohistochemical analysis with this antibody preparation (Fig. 5A). Staining was noted in the epithelial cells of the smaller glands and ducts, coinciding exactly with sites of CRABP(II) expression (Fig. 5B), the putative marker for this system of RA synthesis. No immunostaining was observed when the primary antibody preparation was omitted (data not shown).
Transfection of ALDH6 into MCF-7 Cells Restores RA Synthesis. Absence of expression of ALDH6 in MCF-7 cells suggested that their inability to catalyze the synthesis of RA from retinal was attributable to this difference from the MTSV1.7 cell line and from normal mammary epithelium. When human ALDH6 was transiently expressed in MCF-7 cells, they indeed gained the ability to oxidize retinal to RA (Fig. 6A). ALDH6-transfected cells produced an average of 141 ± 51 pmol RA per 60-mm dish of cells (n = 4), whereas vector-transfected cells produced only 11.4 ± 7.0 pmol RA (n = 5). This established the ability of ALDH6 to carry out this oxidation in a cellular environment in addition to its demonstrated activity in the test tube. Furthermore, ALDH6-transfected MCF-7 cells could make a small but significant amount of RA when provided with retinol, whereas normal MCF-7 cells or vector-transfected cells could not, suggesting the primary defect in RA biosynthesis lies in the second step (Fig. 6B).

As a further test of these results, we observed that, after transfection of ALDH6 into MCF-7 cells, cytosol from these cells gained the ability to convert retinal to RA in the in vitro assay (Fig. 7A) with an average activity of 20.4 ± 4.5 pmol RA/mg protein/min (n = 8). This compares well with an average activity of 27.2 ± 7.1 pmol RA/mg protein/min from MTSV1.7 cytosol (n = 9). This activity was absent in normal or vector-transfected MCF-7 cells, because no detectable RA was produced by incubation of cytosol with retinal. No inhibition of activity was observed with the inclusion of cellular retinol-binding protein in the reaction, a protein that can bind retinal and is found in cells that synthesize RA (data not shown). Expression of ALDH6 in cytosol from transfected cells was further confirmed by Western blot analysis of the cytosolic fraction from transfected cells (Fig. 7B).

DISCUSSION

Our previous work established that normal mammary epithelial cells have the ability to synthesize RA, an ability that was greatly reduced or lost in five of six cell lines derived from human breast cancers (13). An essential question is whether this loss had occurred prior to establishment of these lines, i.e., does loss of the ability to synthesize RA potentially contribute to the development of some breast cancers? This cannot currently be addressed by measurement of RA itself, in biopsy material for example, because of technical difficulties. However, identification of the enzymes involved in the synthesis of RA from retinol will allow molecular biological and immunological techniques to be used to address the question.

Here we identified that the RALDH activity present in an immor-
talized but nontumorigenic human mammary cell line is the previously cloned human ALDH6 enzyme and that this enzyme is expressed in normal human breast epithelia. When transiently transfected into MCF-7 cells that are unable to convert retinal to RA and are deficient in expression of ALDH6, the cells gain the ability to synthesize RA from retinal as well as some ability to synthesize RA from retinol. These findings indicate that ALDH6 is the physiologically relevant enzyme involved in the biosynthesis of RA in mammary epithelium and that lack of expression of this enzyme in a breast cancer cell line was responsible for the inability to synthesize RA from retinol.

However, it should be stressed that synthesis of RA from retinol is a two-step process, with the conversion of retinol to retinal generally considered to be the rate-limiting step. The ability of the MCF-7 cells transfected with ALDH6 to convert retinal to RA was comparable with the ability of the MTSV1.7 cells to do this conversion. However, the production of RA from retinol was markedly less, indicating a potential problem in the first step. Identification of the enzyme responsible for the oxidation of retinol to retinal is necessary before one can consider any analysis of a correlation between loss of RA-synthesizing ability and cancer development. This is a current goal of this laboratory because we have detected none of the known retinol dehydrogenases in a rat mammary carcinoma cell line that is capable of synthesizing RA (NMU line), indicating that the enzyme will be novel.

Several different lines of evidence (reviewed in Ref. 24) suggest that defects in the RA signaling pathway are involved in cancer, from the observation that a diet deficient in vitamin A leads to an increase in the number of tumors in animal models to the observation that RA itself can induce the differentiation and inhibit the growth of many tumor cells, as well as the identification that components of the RA signaling mechanism are absent in cancer cells. Particularly in breast cancer, a number of studies have shown aberrant expression of the nuclear receptors or of binding proteins for retinoids. Decreased expression of the β isofrom ofRAR was noted in several breast cell lines (25–28); the work by Jing et al. (28) also showed that expression of cellular retinol-binding protein was constitutively lower in breast cell lines compared with normal breast tissue. Xu et al. (29) noted that loss of RARβ expression was progressive from normal tissue to premalignant lesions and lowest in invasive cancers. This may be of particular interest with regard to the work here in that RARβ is directly induced by RA (30) and therefore raises the question of whether a defect in synthesis of RA is perhaps the more fundamental defect in some malignant transformations.

The sequence of these events, i.e., loss of RA signaling, loss of receptor expression, and malignant transformation, remains to be elucidated, but to the growing body of evidence linking aberrant RA signaling and cancer we add the loss of ALDH6 expression in MCF-7 cells as the first molecular defect to be identified to explain loss of the RA-synthesizing phenotype in breast cancer cells.

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**REFERENCES**


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