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ORIGINAL ARTICLE

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Differences in uptake and metabolism of retinoic acid between estrogen receptor-positive and -negative human breast cancer cells

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Abstract *Purpose*: Our previous work had shown that retinoic acid (RA) inhibits cell growth and induces apoptosis in estrogen receptor-positive (ER-positive) MCF-7 and T-47D human breast carcinoma cells, but not in ER-negative human breast carcinoma cells MB-231 and MB-453. The purpose of this work was to determine whether these differences might be due to differences in uptake and metabolism of the drug between ER-positive and ER-negative cells. *Methods*: We measured RA uptake in cultured human breast cancer cells and determined its metabolism by high-pressure liquid chromatographic analysis. *Results*: The two ER-positive cell lines reached maximum RA uptake at about 2 h, followed by a sharp decline, so that most RA had disappeared from the cells and from the medium by 24 h and was found as oxidation products in the culture medium. In contrast, the two ER-negative cell lines showed a pattern of lower accumulation without the sharp increase and subsequent steep decline, so that by 24 h there was more RA in these cells and their culture medium than in the RA-responsive ER-positive cells, even though at 2 h the ER-negative cells had taken up less RA than the ER-positive cells. Kinetic analysis of the uptake of RA in MCF-7 cells was consistent with rapid movement across the cell membranes and the actual rate determined by diffusion of albumin-bound retinoid to the cells. *Conclusions*: This study is the first to demonstrate profound differences in RA accumulation and confirms previous results on different rates of RA metabolism between ER-positive and ER-negative human breast cancer cells. The findings reported here, therefore, may introduce additional elements to be considered in the design of new drugs for cancer chemoprevention and therapy.

Key words Free diffusion · Retinoic acid uptake · Breast cancer cells

Abbreviations *DPBS* Dulbecco's phosphate-buffered saline · *ER* estrogen receptor · *FBS* fetal bovine serum · *RA* all-*trans*-retinoic acid · *RARs* retinoic acid receptors · *RXRs* rexinoid receptors

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Introduction

Retinoids have been implicated as chemopreventive and differentiating agents in a variety of systems. Retinoids exert their action by interacting with two families of nuclear receptors, the retinoic acid receptors (RARs), and the rexinoid receptors (RXRs). Retinoic acid (RA) is present in plasma bound to serum albumin, but the source of plasma RA remains uncertain.

We [1] and others [2] have shown that the ER-negative cell lines MB-231 and MB-453 fail to respond to RA, whereas the growth of ER-positive cells MCF-7 and T-47D is inhibited by RA [1], and upon treatment with RA undergo apoptosis and show induction of the enzyme tissue transglutaminase [3]. We have also found that, paradoxically, the ER-negative cells accumulate RA and metabolize it only slowly. In sharp contrast, the ER-positive cells actively metabolize RA, so that the

half-life of the drug is less than 6 h in serum-free medium [1].

We sought to determine whether the kinetics of RA uptake are different between the two types of cells and whether they conform with rapid passive diffusion, as expected from published work [4].

Materials and methods

Reagents

All-*trans*-RA was purchased from Sigma (St. Louis, Mo.). [11,12-^{3}H(N)]all-*trans*-RA (35.8 Ci/mmol) was purchased from NEN (Boston, Mass.). Aquasol and acetonitrile were purchased form Packard Instrument Co., and Burdick and Jackson, respectively.

Cell culture

Uptake was measured in four human breast carcinoma cell lines. ER-positive MCF-7 and T-47D cell lines and ER-negative MDA-MB-231 and MDA-MB-453 cell lines were purchased from American Type Culture Collection (Rockville, Md.). The cell lines were serially passaged as monolayer cultures in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO-BR, Gaithersburg, Md.), plus standard antibiotic/antimycotic solutions (Biofluids, Rockville, Md.). The 75-cm^2 vented culture flasks (Costar, Cambridge, Mass.) were placed in a humidified atmosphere containing 5% CO_2 at 37 °C until near-confluence was achieved as assessed by phase-contrast microscopy. Cells were washed with phosphate-buffered saline (Dulbecco's PBS, DPBS, pH 7.5) without Ca^{2+} or Mg^{2+} and harvested by a 3-min treatment with 0.05% trypsin/0.02% Versene (Biofluids, Rockville, Md.).

Measurement of uptake of [^{3}H]RA

Uptake of radiolabeled RA was performed in 48-well cluster trays (Costar Co., Corning, N.Y.). Cells were seeded at a density of 15,000/well. After 24 h incubation the 10% FBS-containing culture medium (0.2 ml) was replaced with 0.2 ml of 2% or 10% FBS containing the radiolabeled RA (0.5 μCi/ml) used at a final concentration of 30 n*M*. Cells were incubated for 20 and 40 s, 1, 3, 5, 10, 20 and 30 min, and 1, 2, 4, 6, 12, 24 and 48 h or as specified. Following incubation, the cells were washed twice with 200 μl ice-cold DPBS containing 1 μ*M* RA. Ice-cold methanol (50 μl) was used to extract the pellet of cells. The cell-associated radioactivity of the methanolic extracts was then measured using a Beckman liquid scintillation counter (Beckman LS-250, Columbia, Md.). All of these procedures were carried out under yellow light.

HPLC analysis of [^{3}H]RA in human breast cancer cells

MCF-7 and MB-231 cells were trypsinized and plated in duplicate for each time-point at a density of 5×10^5 cells in 60-mm dishes in 5 ml of 10% FBS medium. After 24 h, the medium was replaced with 5 ml of medium containing 2% FBS and either [^{3}H]RA (0.5 μCi/ml; 30 n*M* final concentration in the medium) or 30 n*M* RA alone for determination of cell numbers. Cells were incubated for 2, 6, 24, 48, 72, and 96 h. At each time-point, 20 μl of the medium was tested for radioactivity in Aquasol (5 ml) in a Beckman liquid scintillation counter, and 100 μl was extracted with 0.9 ml methanol. Cells were rinsed with 1 ml ice-cold DPBS containing 30 μ*M* RA, rinsed with DPBS alone, scraped and extracted with 1 ml ice-cold methanol. Extracts were centrifuged for 5 min at 14,000*g*, and the radioactivity was measured. Medium and cell extracts were stored at −70 °C until analyzed by HPLC. The analysis of metabolites was performed on a reverse-phase Partisil 10 ODS-2 column (4.6 mm ID × 25 cm; Whatman, Clifton, N.J.) fitted with a precolumn of Pellicular ODS (Whatman). A Beckman model 110 A pump was connected to a Knauer WellChrom K-2500 spectrophotometer (Sonntek, U. Saddle River, N.J.), connected in series to a Radiomatic radioactivity flow detector (150TR flow scintillation analyzer; Packard Instrument Co., Meriden, Ct.). Acetonitrile and 1% ammonium acetate in water (65:35) was used as the mobile phase, according to the procedure of Frolik [5] at a flow rate 2.2 ml/min.

Results and discussion

Uptake of RA

In previous studies we have observed an unexpected association between the ability of a variety of cell lines to metabolize RA and their sensitivity to growth inhibition by 500 n*M* RA in serum-free medium or in medium containing 0.5% serum [1, 6]. Cell-associated radioactivity was studied for times from 2 to 96 h. Uptake usually was at, or near, maximum values by the first time-point. Further, radioactivity was predominantly associated with authentic RA at 2 h in all cell lines [1]. This experimental design was thus uninformative on the kinetics of the rapid uptake phase. We therefore designed a series of studies to examine the rapid phase of drug uptake and to explore possible differences between two ER-positive (MCF-7 and T-47D) and ER-negative (MB-231 and MB-453) human breast cancer cell lines. At issue are both the rate and extent of uptake of RA.

Effect of serum protein

It is well known that RA is bound to serum albumin [7]. It seemed likely that the serum content of the medium would alter the thermodynamics and kinetics of uptake by cells in culture. Further, the activity of RA may differ so much between typical conditions of incubation with serum-free or low-serum medium and plasma that interpretation of the effects of the drug in vivo may be substantially confounded. It has been reported that in culture medium containing 10% serum the uptake of radioactive RA is reduced compared with the uptake in serum-free medium. The significant difference in metabolism between sensitive and resistant cell lines could not be observed, and an effect of RA on cell growth was not detected [1] until much later, i.e. 72–96 h, probably because of the large amounts of albumin in serum. Other studies have shown that small amounts of serum attenuate the effects of 13-*cis*-RA on small-cell lung cancer cells. Both the induction of RAR-β mRNA and the concentration-dependent effect of the retinoid on cell growth are substantially reduced compared to no-serum conditions [8]. It is also of interest that the addition of RA up to 10 μ*M* in the presence of

bovine serum albumin does not affect the uptake of radioactive RA by human foreskin keratinocytes [9]. The authors concluded that this uptake is a nonspecific and nonsaturable process and the likely cause is the avidity of RA for the cell membrane. They also concluded that serum albumin influences RA uptake and metabolism.

Total cell-associated radioactivity from [^{3}H]RA in MCF-7 and T-47D cells cultured in 2% FBS rose steeply to a maximum within 2 h and then fell rapidly (Fig. 1A,B). In sharp contrast, RA accumulation by the ER-negative MB-231 and MB-453 cells occurred to a lesser extent and without a visible peak (Fig. 1C,D). Cell numbers did not change significantly between ER-positive and ER-negative cell lines during the first 24 h of the experiment. Uptake in 10% FBS was consistently lower within the first few hours. The apparent uptake values presented in Figs. 1, 2 and 3 are probably somewhat high, because no correction was made for adsorption of RA to the plastic culture plate.

The adsorption of RA to the culture wells at 2% and 10% FBS in the absence of cells is shown in Fig. 4.

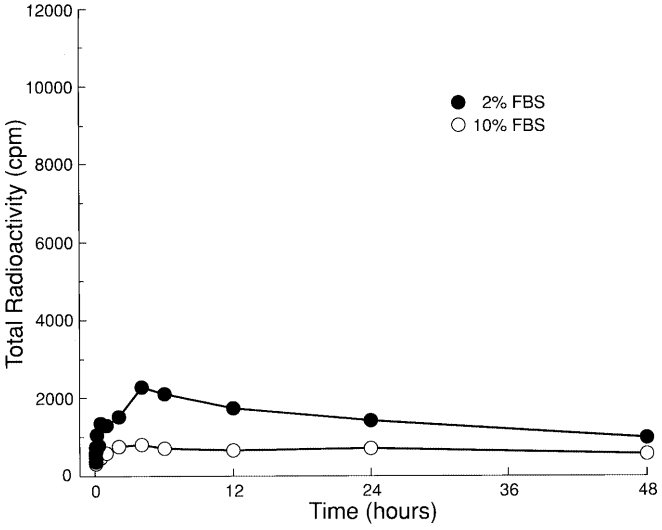
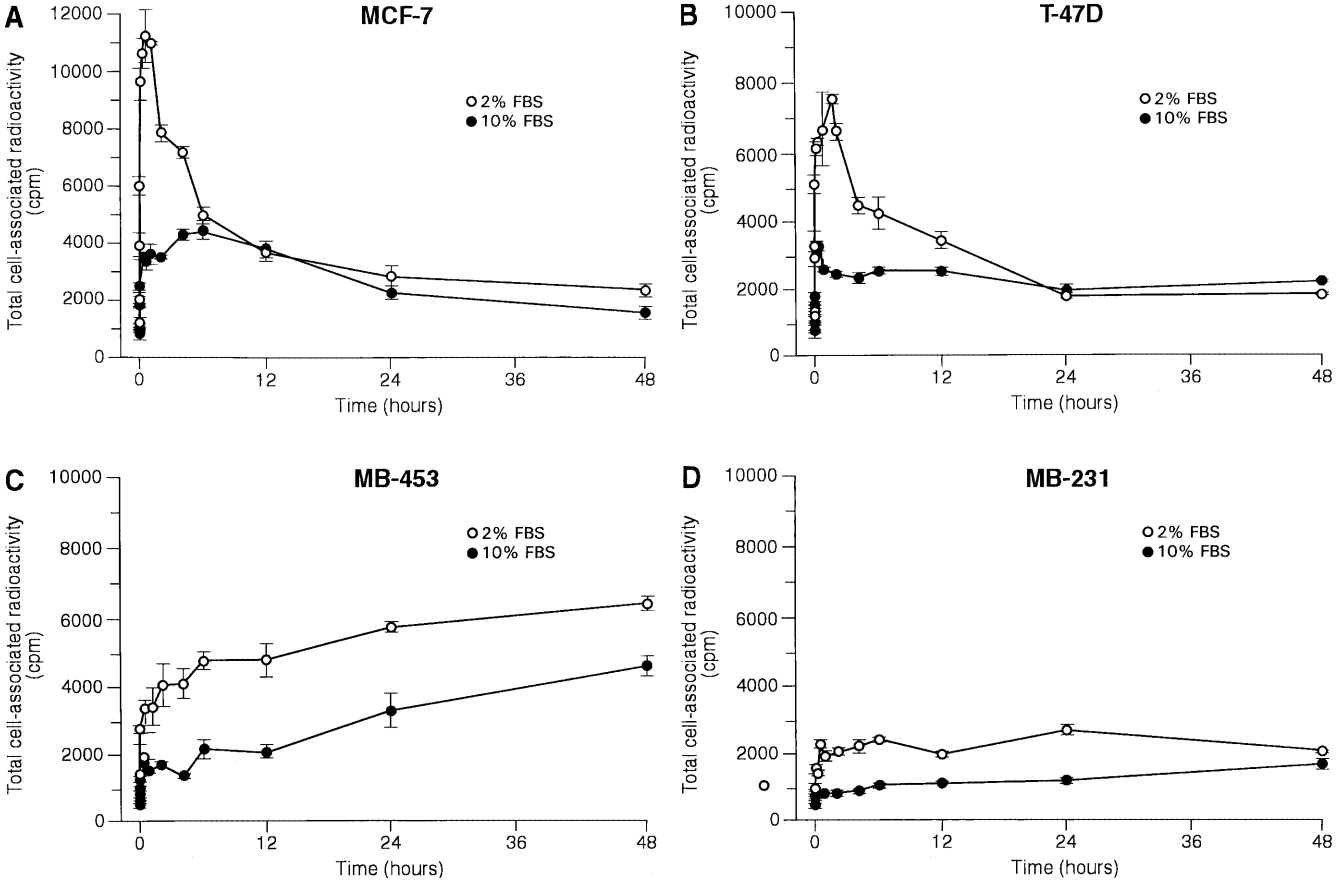


Fig. 2 Plastic culture plate-associated radioactivity as measured under the same conditions as for Fig. 1 with no cells in the wells

Adsorption was not negligible compared with the uptake by the cells, but we elected to present the raw cellular accumulation data without correction for the likely effect of adsorption to the plastic. The rigorous form that the correction would take is not completely clear, and the control adsorption experiments are presented simply as a reference.

Fig. 1A–D Cell-associated radioactivity in human breast cancer cells incubated with 30 n*M* RA in 2% and 10% FBS at 37 °C (**A** ER-positive MCF-7 cells, **B** ER-positive T-47D cells, **C** ER-negative MB-453 cells, **D** ER-negative MB-231 cells). The conditions of incubation and harvesting procedures are described in Materials and methods. Cell layers and dishes were washed with cold buffer containing 1 μ*M* RA prior to harvesting



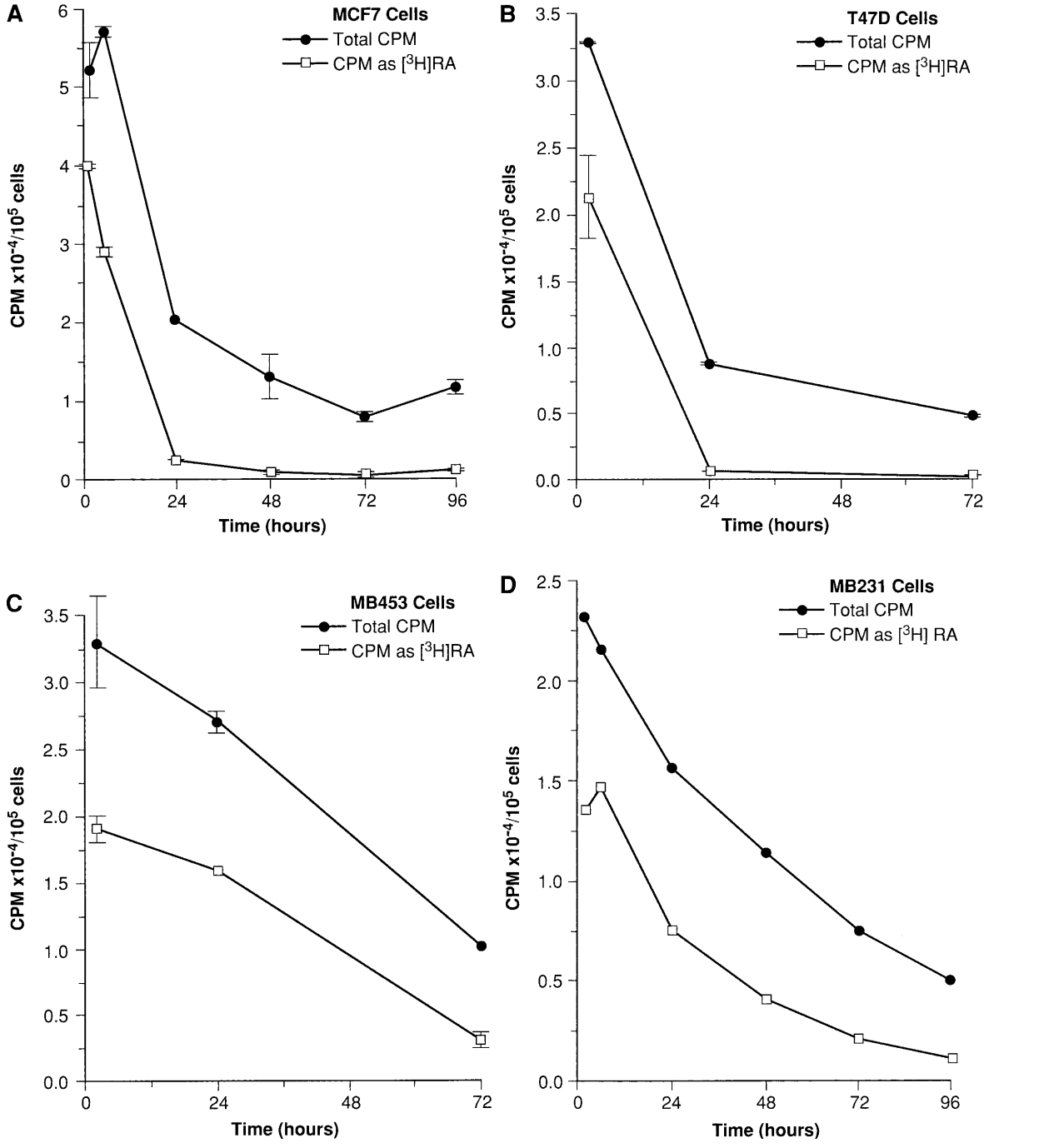


Fig. 3A–D HPLC analysis of intact [^{3}H]RA versus total radioactivity in cell extracts. **A** MCF-7 cells incubated for 96 h. Most of the cell-associated radioactivity after 24 h is in oxidation products. Considering that RA was at a concentration of 30 n*M* and that the volume of a cell is approximately 1.6 pl, a RA concentration of the order of 15 μ*M* is obtained in MCF-7 cells at about 2 h of incubation. **B** T-47D cells incubated for 72 h. **C** MB-453 cells incubated for 72 h. **D** MB-231 cells incubated for 96 h

Next, we studied the extent of metabolism in the cell-associated compartment. HPLC analysis of the methanolic cell extracts of MCF-7 and T-47D cells showed that most (approximately 70%) of the radioactivity within the cells was [^{3}H]RA within the first 2 h. However, most RA had disappeared by 24 h from MCF-7 cells (less than 2,500 cpm/10^5 cells, Fig. 2A) and T-47D cells (less than 1000 cpm/10^5 cells, Fig. 2B). In sharp contrast, radioactive RA persisted well beyond 24 h in MB-453 cells (more than 15,000 cpm/10^5 cells, Fig. 2C) and in MB-231 cells (7,500 cpm/10^5 cells, Fig. 2D). As a consequence of slower metabolism, intact RA was also much higher in the medium from ER-negative MB-231 cells (Fig. 5A) and MB-453 cells (Fig. 5B) than in the medium from the ER-positive MCF-7 cells (Fig. 5A) and T-47D (Fig. 5B) cells. These results are consistent with greater uptake and faster disposition of RA by ER-positive human breast cancer cells.

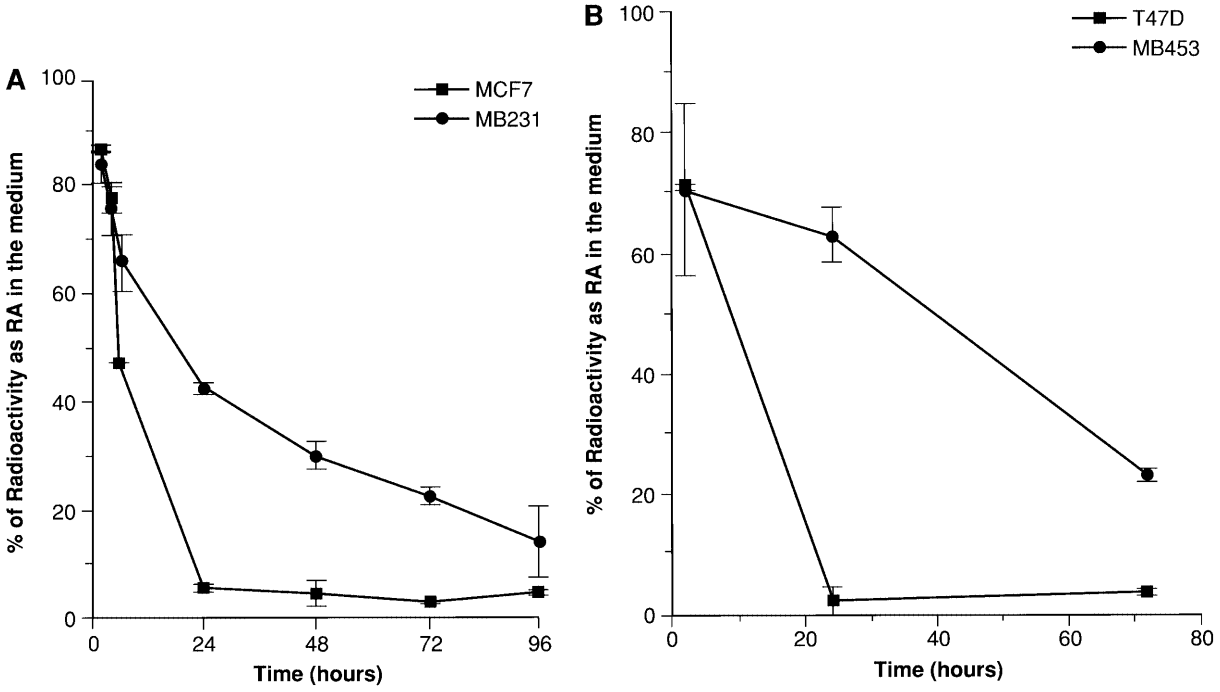
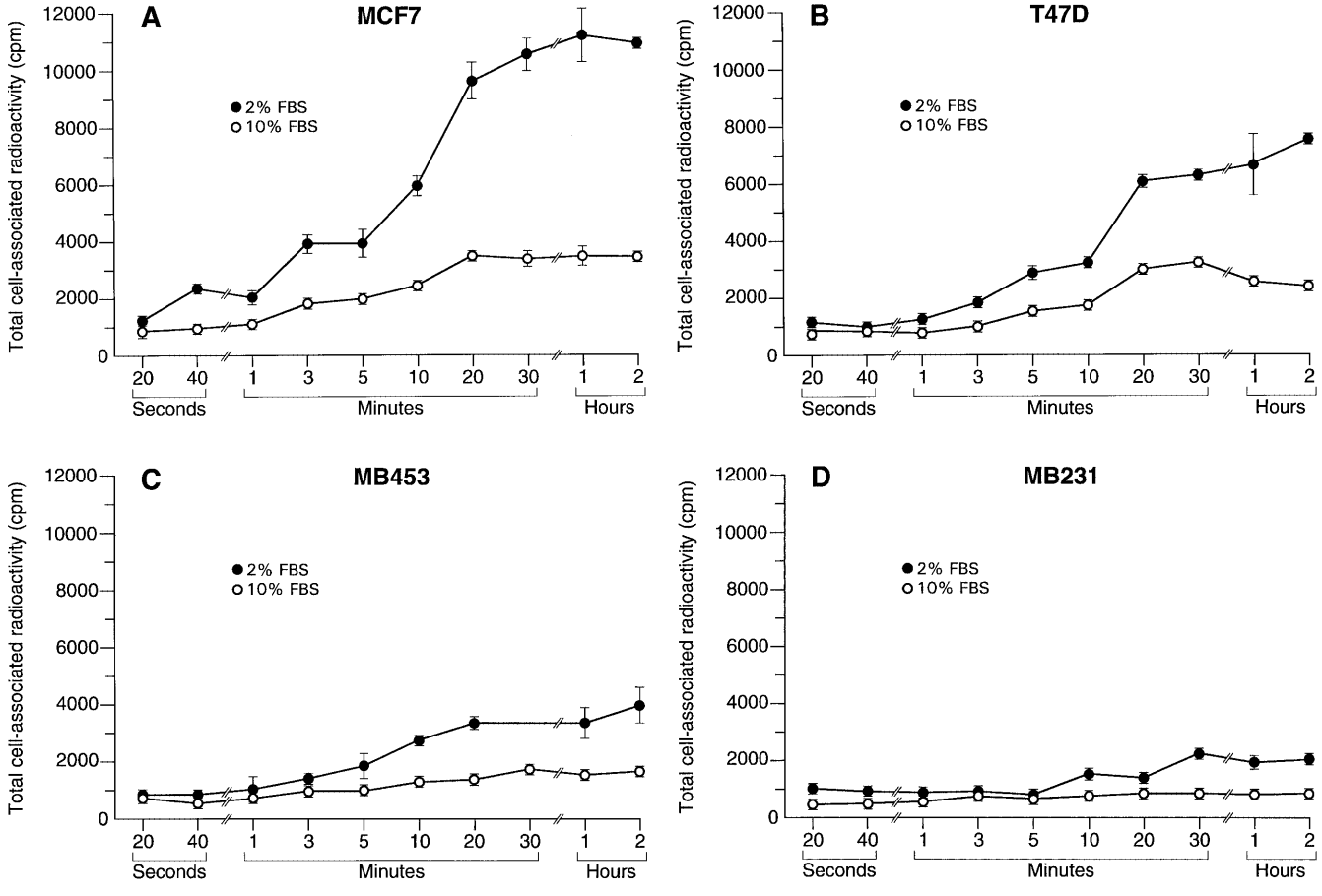


Fig. 4A,B Medium RA reflects cell-associated RA turnover. Medium from MCF-7 (**A**) and T-47D (**B**) cells showed a faster rate of RA disappearance medium from MB-231 (**A**) and MB-453 (**B**) cells

Fig. 5 Short-term uptake of [^{3}H]RA by cultured human breast cancer cells in 2% and 10% FBS



Effect of serum on extent of uptake

Since metabolic studies had shown that the dominant form of radioactivity is still associated with RA in cultured cells for up to 2 h, we examined the radioactivity in the four cell lines (ER-positive MCF-7 and T-47D, and ER-negative MB-231 and MB-453) during the first 2 h of incubation. The results are shown in Fig. 3. Experiments were conducted with both 2% and 10% FBS in the medium. The apparent uptake in the two ER-positive cell lines (Fig. 3A,B) exceeded that in the ER-negative cell lines (Fig. 3C,D) under comparable medium conditions, but the increased serum significantly reduced RA uptake in all cell lines (Fig. 3A–D). Values at 2 h (expressed as percentages of the counts absorbed from the medium) at 2% and 10% FBS, respectively, were approximately: MCF-7, 11% and 3.5%; T-47D, 7.5% and 2%; MB-231, 2% and 0.8%; MB-453, 4% and 1.7%. It is clear that the increase in the FBS content of the medium reduced apparent accumulation by a factor of about three.

Cell-to-medium concentration ratio

The percentage of RA absorbed does not properly describe the distribution of RA between the cells and the medium to which they are exposed. If we assume that the volume of a breast cancer epithelial cell is 1.6 pl, then 15,000 cells have a volume of 2.4×10^{-8} l, while the volume of the medium is 200 µl. Then, for example, if 5% of the RA is absorbed by the cells, the cell-to-medium concentration ratio is $5 \times 200 \times 10^{-6}/95 \times 2.4 \times 10^{-8} = 439$. The apparent distribution values far exceed tissue-to-plasma concentration ratios that have been found in pharmacokinetic studies. Administration of RA to mice by both the intravenous [10] and oral [11] routes produces concentrations in a number of tissues in vivo that are usually within a factor of about two of the serum levels. Some of the large discrepancy between the distribution coefficients of RA into cells in culture and the observed pharmacokinetic distribution coefficients may derive from the intrinsic binding characteristics of the cells or cell populations, but a major portion is probably accounted for by the much larger albumin concentration in whole serum than in the culture medium.

Human serum or plasma has been reported to contain approximately 3–4 g albumin per 100 ml [12]. The FBS was reported by the supplier to contain 2.52 g albumin per 100 ml. Therefore, 2% FBS contains 0.05 g albumin per 100 ml culture medium, and 10% FBS contains 0.25 g albumin per 100 ml. Interstitial fluid would be the corresponding bathing medium in solid tumors. This is neither lymph nor plasma; however, the thermodynamic free concentration of RA may be the same or very similar between plasma and interstitial fluid. The absence of lymphatics in tumors and their relatively permeable vasculature compared with continuous capillaries make the quantitative dynamics of tumor interstitial fluid considerably different from the dynamics of lymph formation. Gullino et al. [13] have reported that the protein content of interstitial fluid of several experimental tumors averages about 33% less than the concentration in serum. The total protein concentration in lymph from the thoracic duct is 52% less than the serum value in rats bearing the Walker carcinoma 256. We have used the tissue-to-serum RA values from pharmacokinetic studies to emphasize that the incubation conditions can have very much less protein than plasma (or tumor interstitial fluid).

Kinetics of absorption

Noy [4] has concluded from biophysical measurements that biological membranes between cells and intracellular organelles are unlikely to provide a significant barrier to RA. This retinoid would be expected to cross membranes rapidly and spontaneously. We defined a mathematical diffusion model in which it was assumed that: (1) the cell membranes are sufficiently permeable to RA that they do not influence the cellular rate of uptake; (2) the actual rate-limiting step is diffusion of RA in association with albumin from the medium to the cell layer at the bottom of the well; (3) convective transport may be ignored; (4) the effective diffusivity of RA is the same as that of albumin (9.3×10^{-7} cm^2/s at 37 °C) [14]; and (5) total radioactivity can be taken to represent RA during the early part of the experiment. We used the graphical solution of the underlying diffusion equation of Crank [15] to predict the time-course of RA accumulation in MCF-7 cells if the thermodynamic equilibrium uptake were 10% in 2% FBS. In Fig. 6 the predicted data are compared with the observed data corrected by subtraction of RA adsorbed by the plastic wells containing no cells. We emphasize that the

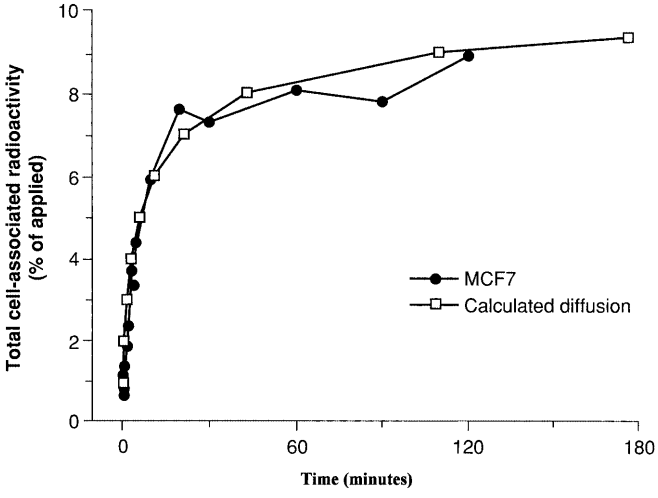


Fig. 6 Comparison between theoretical and measured uptake of [^{3}H]RA by MCF-7 breast cancer cells. The data were obtained in a separate experiment with its own controls for adsorption to the plastic wells

theoretical line in Fig. 6 is an a priori prediction consistent with the assumptions made; it does not utilize the data observed in this experiment other than the estimate of the equilibrium value. If the uptake value of 10% is used, the equilibrium RA concentration in the cells is about 25 μM.

The success of the diffusion model in predicting the observed data is consistent with the first assumption stated above, i.e. that the MCF-7 cell membranes are very permeable to RA. Since the rate of uptake under the conditions of this experiment appeared to be limited by diffusion to the surface of the cells, the experiment did not permit estimation of the actual permeability of the cell membranes or elucidation of the characteristics of membrane transport.

In conclusion, this work demonstrated profound differences in retinoid accumulation between the ER-positive and ER-negative cultured human breast cancer cell lines studied. In addition to differences in accumulation, the two ER-positive cell lines showed greater ability to metabolize RA and most of its metabolites were found in the medium. The two ER-negative cell lines, paradoxically, showed an accumulation of intact RA over longer time periods, probably because of decreased rates of metabolism combined with cell division.

The following important differences between some ER-positive and ER-negative human breast carcinoma cell lines have emerged: in contrast to the relative unresponsiveness of ER-negative cells, ER-positive cells respond to RA by inhibition of cell growth [2, 6], increased apoptosis [3], faster retinoid metabolism [1], induction of transglutaminase II [3] and an increased tyrosine phosphorylation in paxillin and focal-adhesion kinase [16].

Since RARs appear to mediate all these actions of RA [17, 18], it is reasonable to suggest that the differences observed in RA accumulation are also associated with altered nuclear receptor function in ER-negative cells. We will next test the hypothesis that faulty RAR-RXR-mediated signaling and/or reduced RA-specific cytochromes are responsible for reduced intracellular utilization.

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