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Oxidative and Reductive Pathways of Estrogens in Hormone Responsive and Non-responsive Human Breast Cancer Cells *In Vitro*

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In order to measure the formation and degradation rates of estradiol by human breast cancer cells, after assessing the biochemical basis of hormone responsiveness and growth response to estrogens, we considered both responsive, estrogen receptor (ER) positive, and non-responsive, ER-negative, breast cancer cell lines, i.e. MCF7, ZR75-1 and MDA-MB231. To this end, we employed a novel "intact cell" approach which allows us, after 24 h incubation, to analyze several enzyme activities in sequence, concurrently with the monitoring of labeled precursor degradation. Our investigations led to the following evidence: (a) the reductive activity of the 17β-hydroxysteroid oxoreductase (17β-HSOR) appears to be higher than the oxidative only in responsive, ER-rich MCF7 and ZR75-1 cells, as also previously observed by others; (b) this activity is, on the contrary, much lower in MDA-MB231 cells and other unresponsive, ER-poor breast cancer cell lines; (c) conversely, the oxidative activity shows an opposite pattern, being limited in MCF7 and ZR75-1 cells and much higher in MDA-MB231 cells. Overall, a 17β-HSOR reductive pathway prevails in both MCF7 and ZR75-1 cells, whilst the oxidative pathway is prevalent in MDA-MB231 cells, leading to a large formation of estrone that is no further metabolized, at least in the experimental conditions used. Our results may provide a likely explanation of previous data on the different estrogen content of breast tumor tissues.

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INTRODUCTION

Although studies concerning tissue content of steroid hormones have been considerably sparse, in more recent years the importance of the peripheral regulation of metabolism of steroids, precisely their formation, degradation and action at target tissue level, has been repeatedly emphasized [1].

There is general consent that blood uptake and local biosynthesis of active hormones by cancer cells should be considered more significant than their levels in plasma. As a matter of fact, it has been reported that intratumor concentrations of either estradiol (E_2) or estrone (E_1) in human breast cancer are far greater (100 up to 1000 times higher) than their respective plasma levels [2]; this is especially true in the postmenopausal age, that is to say when the highest incidence rate for breast cancer is encountered.

Another piece of evidence comes from the studies of Pasqualini's group in Paris [3]. The authors observed that the estrogen content of human breast cancer tissues is mostly accounted for by conjugate- (mainly sulfate-) E_2 or E_1 more than their free-forms; interestingly, the E_1-sulfate (E_1S) was found to represent the major estrogen component in plasma of normal women throughout the menstrual cycle. Furthermore, previous

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studies [4] revealed a differential accumulation of E_2 and E_1 in human breast cancer tissues. In particular, significantly greater amounts of E_2 were found in estrogen receptor (ER)-rich tumor tissues when compared to E_1 content [2], although other studies have claimed that such an increase is not significant [5].

Most of the previous studies were exclusively focused on both free and conjugate forms of E_2 and E_1. This could, however, be misleading because they disregarded the relevant biological activity exerted, either *in vivo* or *in vitro*, by other estrogen derivatives, wrongly considered in the past as "minor estrogens". For instance, *in vitro* studies have indicated that the more polar catechol-estrogens (CCE) or the 16αhydroxy-E_1 (16αOH-E_1), respectively inhibit or stimulate growth rates of human breast cancer cells [6]. Additionally, higher amounts of 16αOH-E_1 have been detected in tumor than in normal human breast tissues [7].

Formation and degradation of estrogens in target tissues are regulated directly by several key enzyme activities, such as aromatase, sulfotransferase, 17βhydroxysteroid oxoreductase (17β-HSOR) and several hydroxylases. All have been commonly measured using the classical enzymology approach, whereby ER positive breast cancer tissues exhibit much higher 17β-HSOR oxidative activity [8]; conversely, data coming from *in vitro* systems clearly stand for a higher reductive rate in the ER positive human breast cancer cell lines [9].

In order to measure both rates of estrogen formation and degradation in human breast cancer cells, we have investigated either hormone responsive, ER positive (MCF7, ZR75-1), as well as unresponsive, ER negative (MDA-MB231), human mammary cancer cell lines. To this end, we employed a novel "intact cell" approach, using high performance liquid chromatography in the reverse phase mode (RP-HPLC) with radiodetection "on line" [10]. This approach allowed us to study the activities of several enzymes of estrogen metabolism in sequence jointly with the continuous monitoring of degradation rate of a labeled estrogen precursor ($[^3H]E_2$ or -E_1, in our case) in living cells, using variable incubation times (from 30 min up to 72 h) [11].

EXPERIMENTAL

Cell culture

MCF-7 (passage 148), ZR75-1 (passage 86) and MDA-MB231 (passage 15) human breast cancer cell lines were all purchased from American Type Culture Collection (Rockville, MD). Morphological features of both MCF7 and MDA-MB231 cells are illustrated in Fig. 1 (A and B). For routine maintenance, cells were grown on plastic dishes in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), all from GIBCO BRL (Uxbridge, Middlesex, England). Cells were routinely tested for mycoplasma contamination. For all experiments, cells having a narrow range of passage number (MCF7 = 149–156, ZR75-1 = 87–96, MDA-MB231 = 16–24) were used.

Hormone responsiveness

Cells were cultured for a week in RPMI medium supplemented with 10% charcoal treated-FCS (CT-FCS). Subconfluent cell monolayers were rinsed twice in PBS-A, harvested and seeded onto 6-well tissue culture plates (9.5 cm^2, Costar Italia srl, Milan) at a density of 2×10^5 cell/well in phenol red-free RPMI medium supplemented with 5% CT-FCS. After 48 h, cells were incubated for 6 days, with a medium change at day 3, with 0.01 to 100 nM E_2 in absence or presence of 10^{-7} M ICI-182, 780, a pure synthetic antiestrogen; control wells received vehicle (ethanol 0.1%) alone. After incubation, [methyl-^{3}H]thymidine (sp. act. 248 GBq/mmol, DuPont de Nemours Italiana spa, Milan) was added (74 kBq/well) in RPMI for 6 h. After the pulse, medium was discarded and cells fixed in methanol for 10 min at -20°C to minimize cell losses. Cells were then washed three times using 2 ml of ice-cold 10% TCA for 10 min. Cell monolayers were therefore solubilized using 1 ml of 0.1% SDS in 0.3 N NaOH and duplicate aliquots (400 μl) of acid-precipitable material were counted for incorporated thymidine in an LS 1801 β-counter (Beckman Inc., Irvine, CA, U.S.A.).

Immunocytochemistry of estrogen and progesterone receptors

Presence of both ERs and progesterone receptors (PgRs) was investigated in MCF7, ZR75-1 and MDA-MB231 cells using the commercially available Abbot (Divisione Dianostici, Rome, Italy) ER-ICA and PgR-ICA kits, as extensively described elsewhere [12]. Briefly, cells were grown directly onto 2-well Lab-Tek Tissue Culture chamber slides (Nunc, Naperville, IL) until 60–80% confluent. Analysis of distribution and intensity of the receptor staining was carried out on a minimum of 50 randomly selected fields by using the Q-ER/PgR software for CAS 200 Image Analyzer (Becton-Dickinson Italia spa, Milan), which automatically yields percent of positively stained nuclei and measures the intensity of staining; the latter was defined as the sum of total optical density for the positive receptor nuclear area over the sum of total optical density of all the nuclei expressed as a percentage.

Radioligand binding assay

ER content was determined by means of radioligand-binding assay, using dextran-coated charcoal and filtration methods to separate bound from unbound ligand in soluble and nuclear fractions, respectively. Cells were harvested and homogenized as extensively

described elsewhere [13]. Cell homogenate was spun at 800 *g* for 5 min at 4°C to separate the soluble (supernatant) from the nuclear (pellet) fraction as routinely carried out in our laboratories [14]. Assay data were analyzed and processed using Scatchard analysis with a software (Oncolog 2.3) we have previously established

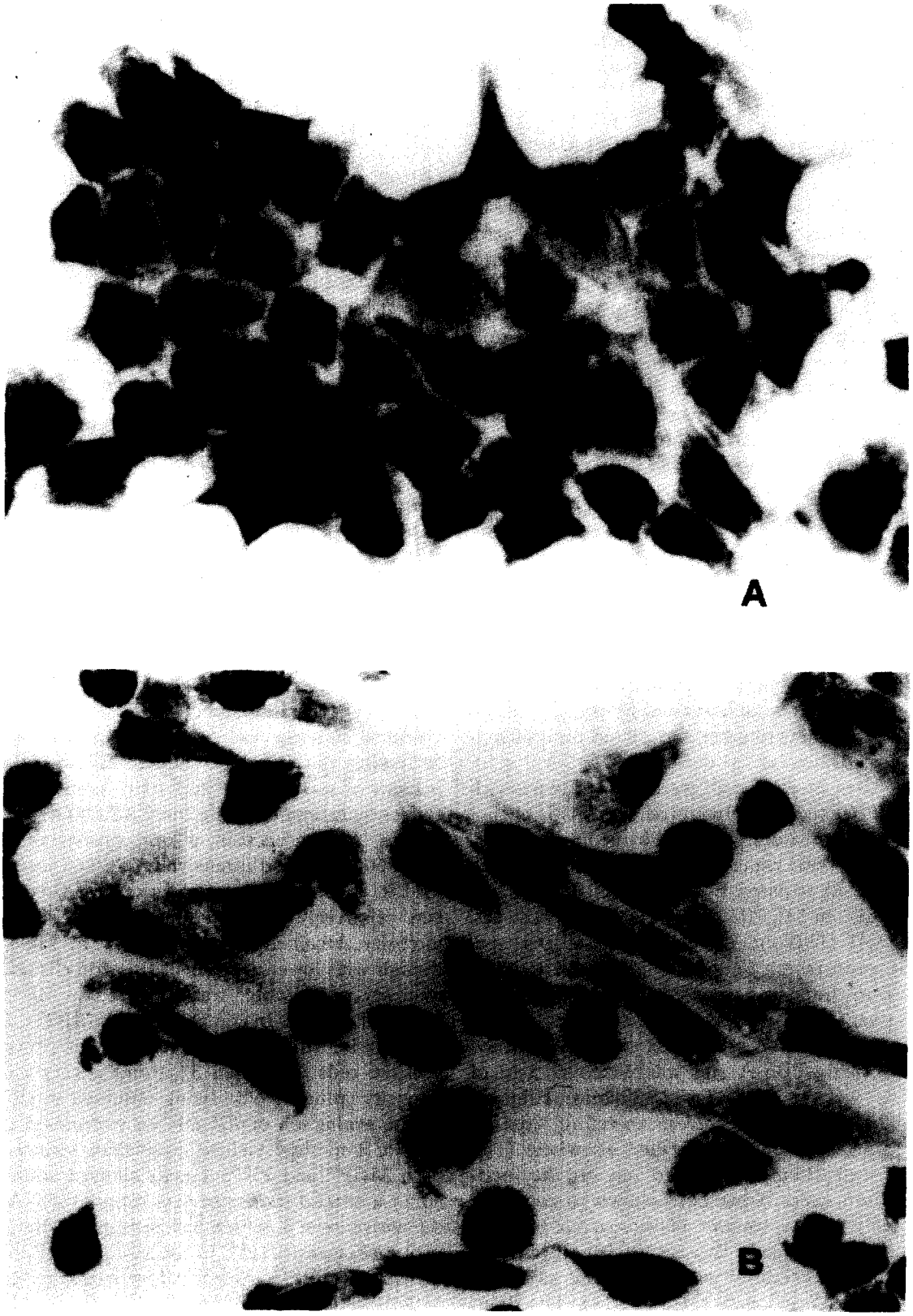

Fig. 1. Morphological aspect of MCF7 (A) and MDA-MB231 (B) cells in culture (×160, May-Grunwald-Giemsa).

[15], yielding both the dissociation constant (K_d) and concentration values (fmol/ml homogenate) of receptors; the latter were expressed as either fmol/mg protein or DNA, for any cell compartment. Data were also analyzed using our model for one or two binding sites, depending on the best fitting achieved. Protein and DNA cell contents were determined using the Bradford [16] and the modified Burton [17] methods, respectively.

Reverse transcriptase-polymerase chain reaction (RT-PCR) of ER

The expression of ER mRNAs in human breast cancer cells was investigated using an RT-PCR system, as recently established in our laboratories [18]. Briefly, total RNA extracted from MCF7, ZR75-1 and MDA-MB231 cells was reverse transcribed and PCR-amplified using a sense primer complementary to a sequence in exon 3 and an antisense primer complementary to a sequence in exon 6 of the human ER gene. Amplification products were then separated on a 1.4% agarose gel and blotted onto nylon membranes. Filters were finally hybridized through Southern blotting techniques using a human ER cDNA, generously donated by Pierre Chambon (University of Strasbourg, France), as a probe.

Estrogen metabolism

The methodological approach and procedures used to measure metabolic pathways of estrogens in *in vitro* systems have been previously optimized [19] and established [10, 20]. Cells were harvested by trypsinization, counted in a haemocytometer and plated onto 60 mm cell culture dishes at a density of $0.5–1.0 \times 10^6$ cells/dish. After 24–48 h, cells were washed twice with PBS-A and the medium substituted with FCS, phenol red-free RPMI medium. After 24 h medium was replaced with the same experimental medium containing $1.0–6.0 \times 10^{-9}$ M tritiated E_2 ([6,7-^{3}H(N)]E_2; sp. act. 42.7 Ci/mmol) or E_1 ([1,4,6,7-^{3}H(N)]E_1; sp. act. 71.5 Ci/mmol) (DuPont de Nemours Italiana spa, Milan) as precursors. Following either 24 or 72 h incubation, medium was transferred to sterile plastic tubes (Costar, Cambridge, MA) and stored at −80°C until estrogen extraction procedure; cells were washed three times using PBS-A and solubilized in 3 ml of SDS 0.1% w/v at 37°C for 15–30 min. Aliquots (100 µl) of the cell lysates were therefore used to estimate DNA content, as described elsewhere [11].

Estrogen extraction was carried out on the incubation medium, since it has been shown to contain a proportionally higher amount of radioactive steroids with respect to the cells themselves [9]. Details of the extraction procedure and extraction efficiency values have been previously reported [11]. The dried extracts were stored at −20°C until chromatographic analysis.

Chromatographic analysis

Extracts were chromatographically analyzed in RP-HPLC, using a Model 324 HPLC (Beckman Instr. Inc., Berkeley, CA) equipped with UV detector, set at 280 nm, and "on line" IC Flo-One/beta three-channel radiometric detector (Radiomatic Instruments, High Wycombe, U.K.). Estrogens were separated under isocratic condition using a Spherisorb ODS-II column (Aldrich Chimica, Milan, 250 × 4.6 i.d. mm), at 20 ± 0.5°C. A computer-aided optimized mobile phase [19, 20], consisting of acetonitrile:0.5 M citric acid (40:60, v/v), at a flow rate of 1 ml/min, was used to separate a wide range of estrogen metabolites within a total analysis time of 20 min. Radiometric detection was performed using a 2.5 ml flow cell and Ready-Flow III (Beckman Analytical spa, Milan) scintillation mixture at flow rate of 6 ml/min. Routine data integration was achieved by a Flo-One/beta F1B IC program (Radiomatic, Tampa, FL) and computed in net cpm after correcting for both sample residence time and background subtraction.

RESULTS

In order to inspect the hormone-sensitivity status of either responsive MCF7 and ZR75-1 or non-responsive MDA-MB231 breast cancer cells, we have used immunocytochemical and radioligand binding assays, as well as the RT-PCR method, to evaluate the expression of ER proteins and mRNA transcripts in these cells.

Immunocytochemical assays of both ER and PgR in MCF7 cells are illustrated in Fig. 2 (A and B). Cytochemical staining was revealed using rat monoclonal antibodies raised against human ER and PgR; both percent of stained cells and stain intensity were subsequently estimated using the CAS 200 image analysis system. As can be seen, a large proportion of nuclei (over 60%) stained intensively (>80% positive stain) for ER; conversely, the number of stained cells for PgR was relatively lower (>30%, 60%), but the stain intensity remained high (>60%). The same pattern, though with lower number of stained cells and weaker intensity of staining, was observed in ZR75-1 cells (data not shown). In contrast, MDA-MB231 cells were negative for either receptor, a very low proportion of positive cells (less than 3%) and a very weak stain intensity (roughly 6%) being observed in all cases.

The evaluation of ER mRNA expression using the RT-PCR method yielded equivalent results. In fact, both MCF7 and ZR75-1 cells showed abundancy of either a normal transcript or a variant mRNA, lacking the entire exon 4, which has been originally identified in our laboratories [18]. In contrast, neither normal nor variant ER mRNA transcripts were detected in MDA-MB231 cells.

Using radioligand binding assay, both MCF7 and ZR75-1 cells exhibited soluble and nuclear

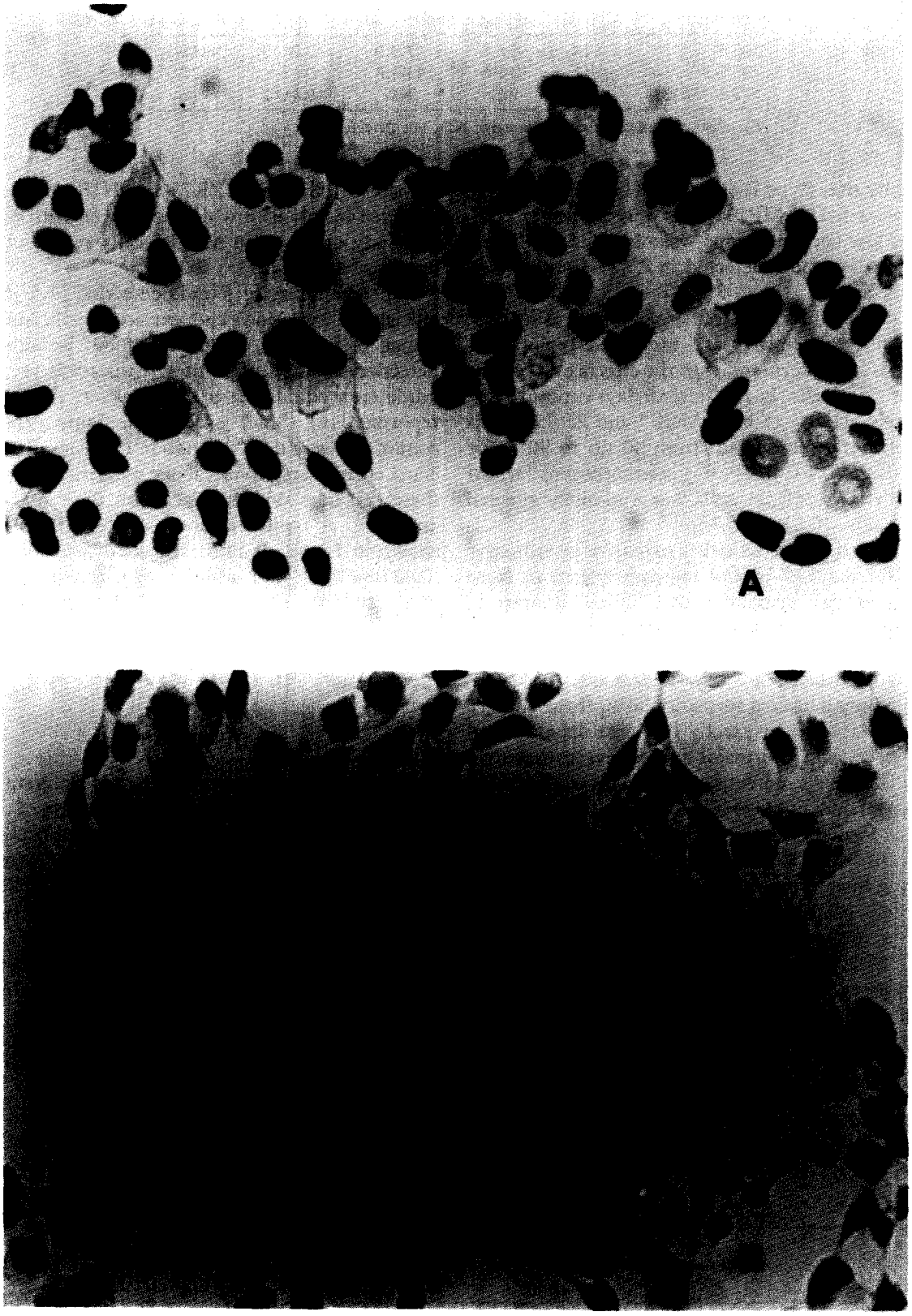

Fig. 2. Immunocytochemical staining for ER (A) and PgR (B) of MCF7 cells using ER-ICA and PgR-ICA methods (× 160).

Table 1. High-affinity estrogen binding sites in human breast cancer cell lines

	Soluble fraction			Nuclear fraction	
Cell line	K_d (nM)	fmol/mg P	fmol/mg DNA	K_d (nM)	fmol/mg DNA
MCF7	0.25	135.1	1796.0	0.28	485.9
ZR75-1	0.52	107.0	1378.8	0.12	292.6
MDA-MB231	ND	ND	ND	0.44	173.6

K_d, dissociation constant; P, protein; ND, not detected.

high-affinity sites (type I) of estrogen binding, whilst in unresponsive MDA-MB231 cells only a nuclear component at low concentrations was observed (50% and 70% lower than those detected in ZR75-1 and MCF7 cells, respectively).

These data are in accord to those obtained using both the immunocytochemistry and molecular biology approaches and, furthermore, with the effects of E_2 on the growth of these cells. In fact, after 6 days exposure, the proliferative activity of both MCF7 and ZR75-1 cells was significantly stimulated (from 30 up to 90%) by physiological (0.1–1 nM) E_2 concentration, whilst growth of MDA-MB231 cells was minimally affected by any E_2 dose (0.01–100 nM).

We have also inspected either oxidative or reductive pathways of the 17β-HSOR enzyme system in these cell lines, through incubation of a limited number of cells ($0.5–1.1 \times 10^6$) with a physiological concentration of a tritiated precursor (E_2 or E_1). As shown in Table 2, 24 h incubation of MCF7 cells with $3–6 \times 10^{-9}$ M E_2 resulted in only a limited formation of E_1 (below 10%). On the contrary, under exactly the same experimental conditions, E_1 was the major radioactive estrogen in MDA-MB231 cells, corresponding to more than 60% of E_2 conversion over 24 h. On the other hand, no conjugate formation could be detected in MDA-MB231 cells, while it ranged from 5 to 15% in MCF7 cells, being lower when compared to that observed in ZR75-1 cells (data not shown).

Concerning the E_1 conversion rates in breast cancer cells, we observed an inverse metabolic pattern. In fact, MCF7 cells exhibited a reduction of E_1 to E_2 3-fold greater than that found in MDA-MB231 cells (10% vs 3% of E_2 formed at 24 h), using exactly the same experimental conditions. In this case no conjugate formation was detected in either cell line. Again, the estrogen conversion metabolic patterns at 72 h confirmed those observed at 24 h in both cell lines (data not shown). Similarly, ZR75-1 cells showed patterns of estrogen metabolism which agree well with both those presently observed in MCF-7 cells and results of our previous studies [21].

Reproducibility of β-radiodetection (2.6 –5.1% coefficient of variation) and extraction efficiency values (range of 90–98%) were very good; in addition, the detection sensitivity limit for tritium was very low (50 crude cpm equivalent to 2.4 fmol), as also previously reported [10]. Since exactly the same experimental conditions were used throughout, the significant differences encountered in metabolic patterns of E_2 and E_1 by these cell lines do not depend on the methodological approach. A typical radioactive RP-HPLC profile is shown in Fig. 3. It illustrates a 10% formation of E_2 (0.42 pmol/ml) after addition of tritiated E_1 precursor (5.0×10^{-9} M) to 5×10^5 MCF7 cells for 24 h; no formation of other metabolic products was observed in this case, nor was there detectable conjugate production.

DISCUSSION

Overall, the present study strongly suggests that two distinct and differently regulated pathways of the estrogen 17β-HSOR exist in cultured human breast cancer cells. As summarized in Fig. 4, using "intact cell analysis", the reductive pathway of 17β-HSOR appears to be higher in the ER positive, while the oxidative pathway is largely prevalent in the ER negative breast tumor cell lines. This eventually leads to accumulation of E_2 in MCF7 cells, whereby this estrogen is poorly oxidized to E_1 or it is converted to conjugate forms, whilst E_1 is reconverted to E_2 at a comparatively higher rate (1:2 ratio of oxidative to reductive 17β-HSOR activity).

On the contrary, metabolic pathways of estrogens are drastically oriented towards oxidation in MDA-MB231

Table 2. Levels of free- and conjugate-estrogens in human breast cancer cell lines

	MCF7				MDA-MB231			
Precursor	E_2	E_1	Conjug	%EE	E_2	E_1	Conjug	%EE
[^{3}H]E_2	2.54 ± 0.13	0.14 ± 0.01	0.12 ± 0.02	96.0 ± 1.0	1.26 ± 0.13	2.00 ± 0.22	ND	98.2 ± 0.8
[^{3}H]E_1	0.39 ± 0.04	3.68 ± 0.23	ND	90.8 ± 1.0	0.13 ± 0.01	4.52 ± 0.41	ND	95.0 ± 0.5

Cells ($0.5–1 \times 10^6$) were incubated with $3–6 \times 10^{-9}$ M labeled E_2 or E_1 as precursors, in FCS, phenol red-free RPMI medium for 24 h; the incubation medium was processed as described in the Experimental section. Values are mean ± SD values (pmol/ml) of six experiments each performed in triplicate. ND, not detectable; Conjug, conjugate.

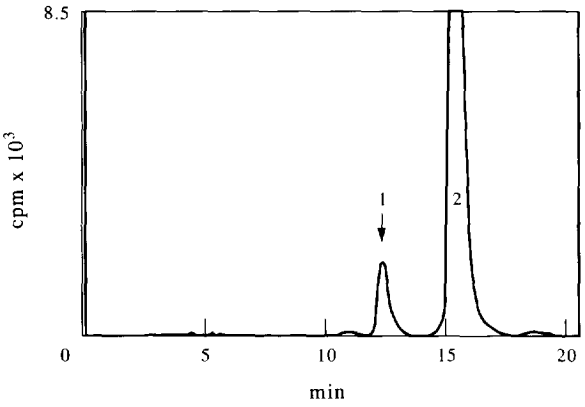

Fig. 3. Estrogen metabolic profile in RP-HPLC and radiometric detection following 24 h incubation of MCF7 cells (5.1×10^5) with [^{3}H]E_1 (5×10^{-9} M). Peak identification numbers and integrated crude cpm values were: (1) = E_2, 11,816; (2) = E_1, 100,653.

cells, where E_2 is quickly converted to E_1, while only a little reconversion of E_1 to E_2 occurs (20:1 ratio); this may result in a noticeable accumulation of E_1, which is apparently no further metabolized, at least in the present experimental conditions. This evidence is also reinforced by results of additional experiments extended up to 72 h incubation.

The significant differences observed in patterns of estrogen metabolism in these *in vitro* systems could not be ascribed to a diverse endogenous content of estrogens, since serum-starvation of cells for 1 week paradoxically enhanced the differences observed between responsive and unresponsive breast cancer cells (not shown).

Data coming from classical enzymology studies suggest that both oxidative and reductive activities of 17β-HSOR are significantly greater in hormone responsive, ER positive breast cancer tissues and cells [8, 22]. This evidence is, however, contradicted by the present estimation of either products' formation or precursor degradation of estrogens in log-phase growing mammary cancer cell lines.

However, we must bear in mind that: (a) the intact cell analysis of a pure epithelial component can be in no way compared to a whole tissue analysis; (b) many

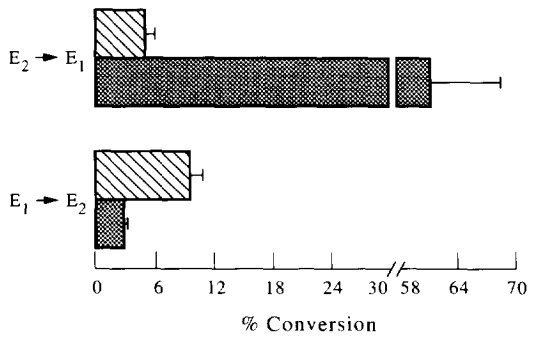

Fig. 4. Oxidative versus reductive activity of 17β-HSOR in MCF7 (hatched bars) and MDA-MB231 (dotted bars) human mammary cancer cells after 24 h incubation.

striking differences separate the two approaches, where the homogenized-cell method, by definition, is featured by an excess of precursor added and affected by many other artifacts, likewise pH, temperature, addition of cofactors, short observation times and, last but not least, disruption of subcellular compartmentalization. All these conditions may well account for the conflicting results, as also previously considered [11].

Although the present data are on one hand strongly at variance with those from classical enzymology, on the other hand they are in good agreement with most previous observation, obtained either *in vitro* or *in vivo* [2, 4, 5]. As matter of fact, prolonged retention of E_2 in MCF7 cells [23], different degradation rates of E_2 in several human cancer cell lines, implying a significantly higher 17β-HSOR oxidative activity in hormone unresponsive, ER negative cells [9], and, on the contrary, equivalence between oxidative and reductive pathways in MCF7 [24] and differential distribution of both E_2 and E_1 in ER positive and negative breast cancer cells [3], have all been reported.

The possible inferences that can be drawn in the light of the present results are, however, compounded by further evidence of a signficant association between other enzyme activities, such as sulfo-transferases and 2-hydroxylases, with both levels of cellular ER and response to estrogens; in particular, a differential formation, even in short-term experiments, of either estrogen-sulfates or 2-hydroxy-estrogens has been detected in the estrogen responsive as opposed to unresponsive breast tumor cells. An apparent production of 16αOH-E_1 was never observed in either cell type (Castagnetta *et al.*, in preparation); this, however, could be strictly connected with the methodology used here and the physico-chemical features of this peculiar estrogen [25].

Therefore, data by our own and other research groups may provide a likely explanation of previous data on the different accumulation of estrogen in breast tumor tissues [2, 4, 7, 8, 26], as also reflected in the estrogen excretion profiles [27].

However, mechanism(s) of such a close association between the ER status and/or hormone responsiveness with pathways of either 17β-HSOR or other key enzyme activities of estrogen metabolism in human breast cancer cells remain unclear and deserve further investigations.

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