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Report

Estrogen responsiveness of IBEP-2, a new human cell line derived from breast carcinoma

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Summary

IBEP-2, an established cell line recently derived from breast carcinoma, was characterized with regard to estrogen receptor (ER) expression, cell mitogenic response to estrogenic stimulation and sensitivity to antiestrogens. In addition, we examined ER modulation following binding of agonist and antagonists, and the ER-mediated induction of progesterone receptor (PgR). ER level in IBEP-2 cells, determined by enzyme-linked immunoassay (EIA), was slightly higher than that measured in MCF-7 cells (662 v.s. 595 fmol/mg protein). When tested on IBEP-2 and MCF-7, various agonists stimulated cell growth with EC50's reflecting different estrogenic potencies ($E_2 \approx$ diethylstilbestrol $> E_1 >$ genistein). IBEP-2 appeared slightly more sensitive than MCF-7, especially to E_2 (at least 4-fold difference between EC50 values). By contrast, IBEP-2 and MCF-7 were equally sensitive to the growth inhibitory effect of antiestrogens 4-hydroxy-tamoxifen (OH-Tam) and ICI 182,780. As revealed by immunoblotting and immunofluorescence using anti-ERα antibodies, ER expression in IBEP-2 cells was modulated by E_2 and estrogen antagonists like it has been shown in other ER-positive cell lines, that is, E_2 and ICI 182,780 caused ER downregulation, whereas OH-Tam induced ER accumulation. Ligand-induced downregulation of ER involved degradation in proteasomes, since it was suppressed by the proteasome inhibitor MG-132. Exposure of IBEP-2 cells to E_2 resulted in a marked (at least 25-fold) induction of PgR, documented by EIA, immunoblotting and immunofluorescence. PgR induction due to E_2 was not modified by MG-132. Interestingly, MG-132 alone produced an ER-independent increase of PgR expression. IBEP-2 might prove to be valuable to study ER-mediated induction of PgR.

Introduction

Epidemiological and clinical studies have clearly established that estrogens are amongst the major factors contributing to the etiology of human breast carcinoma [1, 2]. Moreover, a sizeable proportion of breast cancer cases respond favorably to a deprivation of estrogen stimulation. Hence, hormonal therapy based on estrogen antagonists is extensively used for the treatment of these neoplasms [3, 4]. Like other steroid hormones, estrogens exert most of their effects on target cells by essentially acting at genomic level *via* intracellular cognate receptors (estrogen receptors, ER) which function as hormone-dependent transcription regulators. The ER status in breast tumors is considered as a reliable prognostic factor with regard to the response to antiestrogen-based hormonotherapy.

Human cell lines derived from breast carcinoma have proven to be particularly useful for the *in vitro* study of molecular mechanisms underlying the hormonal regulation of tumor cells by estrogens. They have also become essential tools for assessing the potency of various antiestrogens. Despite the numerous reports describing established cell lines derived from

human mammary cancer (reviewed in [5] and more recently in [6]), it is amazing that studies devoted to ER-mediated cell signaling and ligand-induced receptor regulation in breast carcinoma rely on a small number of cell lines, amongst which the ER-positive cell lines MCF-7 [7] and T-47D [8], and the ER-negative cell lines of the MDA series [9] are the most extensively utilized as *in vitro* models. Yet, these permanent cell lines are only representative of a few clinical cases. Moreover, since they have been subcultured for more than two decades, they might have somehow evolved and diverged from the phenotype of the neoplasms of origin. Indeed, cell immortalization *in vitro* can occasionally be accompanied by dedifferentiation (e.g., [10]).

In this context, a recent report has described the development of three cell lines established from malignant pleural effusions of patients with invasive breast cancer [11]. Of these cell lines two were found to express progesterone receptors (PgR) (IBEP-1 and IBEP-3) but were ER-negative, whereas the third was PgR-inducible and ER-positive (IBEP-2). Although the IBEP cell lines have been systematically characterized with respect to morphology, expression of lineage markers and growth properties, little is known concerning their reactions to the hormonal environment, in particular the response of IBEP-2 to estrogenic stimulation. Therefore, we undertook this study to evaluate the sensitivity of IBEP-2 to estrogen agonists and antagonists (proliferation assay), using as a reference the well-characterized MCF-7 cell line. IBEP-2 was also tested for ER modulation caused by 17β-estradiol (E_2) and antiestrogens. Furthermore, we assessed in this cell line the induction of PgR by E_2. Finally, since ligand-induced ER degradation in MCF-7 and other cell lines has been reported to occur in proteasomes [12–18], we examined the involvement of these molecular complexes in ER downregulation in IBEP-2 cells.

Methods

Drugs

Estrone (E_1) and diethylstilbestrol (DES) came from Sigma-Aldrich (Bornem, Belgium). Genistein was purchased from Alexis (Lausen, Switzerland). 17β-estradiol (E_2), 4-hydroxy-tamoxifen (OH-Tam) and MG-132 were supplied by Calbiochem-Novabiochem (La Jolla, CA). ICI 182,780 was obtained from Tocris Cookson (Bristol, UK). Stock solutions of these compounds were prepared at least 10,000-fold more concentrated in ethanol (for estrogens, antiestrogens and genistein) or dimethylsulfoxide (for MG-132), and stored at −20°C.

Cell culture

IBEP-2 cell line was established from the pleural effusion of a patient with advanced breast carcinoma, as described previously [11]. MCF-7 cell line (ATCC no. HTB22) was originally obtained in 1977 from the Michigan Cancer Foundation (Detroit, MI). Routine cell propagation and experimental studies were carried out at 37°C in a cell incubator with humid atmosphere at 5% CO_2. Unless specified otherwise, cells were cultured in T-flasks containing Dulbecco's Modified Essential Medium (DMEM, BioWhittaker Europe, Verviers, Belgium) supplemented with Phenol Red, 10% fetal bovine serum (FBS, HyClone, Logan, Utah), 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (DMEM-FBS) (supplements from BioWhittaker or Gibco^{TM}, Invitrogen, Merelbeke, Belgium). Cells were passed once or twice a week, with a renewal of the medium every 2 days. For subculture and measurement of growth, the cell monolayers were rinsed with Dulbecco's phosphate buffered saline (DPBS) and cells were dislodged from the vessel bottom by treatment with trypsin-EDTA solution. After vigorous pipetting, concentrations of cells in suspension were determined in an electronic cell counter (model Z1 Coulter counter). For routine cell maintenance, IBEP-2 and MCF-7 cells were plated in 75- or 25-cm^2 T-flasks at a density of 10^4 cells/cm^2. Before measurement of cell growth and demonstration of steroid receptors by immunofluorescence, Phenol-Red-free DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, Utah), 25 mM HEPES and 2 mM L-glutamine (estrogen-free medium, EFM) was substituted for DMEM-FBS and cells were grown in this medium for a minimum of 2 days.

The effects of estrogen agonists and antagonists on cell growth were assessed as described previously [19], with minor modifications. Cells in EFM were plated in 12-well dishes at a density of 10^4 cells/cm^2. At day 1, the seeding medium was replaced by fresh medium (EFM) containing estrogen agonists or antagonists at concentrations specified in "Results". Cells

were trypsinized at day 4 and counted as described above. Solutions of estrogens and antiestrogens in culture medium were prepared extemporaneously from stock solutions. IC_{50}'s referred to drug concentrations producing half maximal inhibition of growth.

Receptor immunostaining

IBEP-2 cells or MCF-7 cells in EFM were plated at densities of 0.5–1 × 10^4 cells/cm^2 on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were fed fresh EFM containing estrogen, estrogen antagonists and/or MG-132 at the concentrations indicated in 'Results'. After the treatment duration specified in 'Results', cell monolayers were rinsed with DPBS and fixed with 4% paraformaldehyde (PAF) in the same buffer. Following fixation, PAF was changed for DPBS where cell cultures were stored at 4°C until immunostaining which was performed within the next 20 h.

Demonstration of ER and PgR by immunofluorescence was achieved as detailed in a previous publication [19]. In short, cells monolayers were rinsed several times with phosphate buffered saline (PBS, 0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.12 M NaCl, pH 7.2) containing 0.2% Triton X-100. For all subsequent incubation and rinsing steps, Triton X-100 was included in buffer to ensure cell permeabilization. Before exposure to primary antibodies, cells were preincubated for 20 min in PBS containing 5% normal goat serum (PBS-NGS) and 0.05 M NH_4Cl to prevent non-specific adsorption of immunoglobulins. Cells were exposed for 60 min to primary antibodies, that is, H-184 (rabbit polyclonal antibody raised against residues 1–184 of human ER-α), HC-20 (rabbit polyclonal antibody raised against residues 576–595 at the carboxy terminus of human ER-α) or C-19 (rabbit polyclonal antibody raised against residues 915–933 at the carboxy terminus of human PgR and detecting both A and B isoforms) (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:40 in PBS-NGS. Thereafter, the cell preparations were incubated for 30 min in presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit immunoglobulins (EnVision™, Dakopatts, Glostrup, Denmark). The next step consisted in a 30-min incubation with rabbit anti-peroxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), followed by fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins antibodies or biotinylated swine anti-rabbit immunoglobulins antibodies (both from Dakopatts) for a further 30 min. Texas Red labeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Pierce Chemicals Co., Rockford, Il). After final rinses in PBS, the coverslips were mounted on glass slides using commercial anti-fading medium (Vectashield®, Vector Laboratories, Burlingame, CA). Negative controls were produced by omitting the primary antibody. This modification resulted in a virtual disappearance of the signal.

The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Excitation wavelengths of 490 and 596 nm and emission wavelengths of 525 and 615 nm were used for the observation of FITC and Texas Red fluorescence, respectively. The appearance of immunostained cell preparations was documented either by taking pictures on color slide films with a conventional photo camera (Leica MPS 60), or by using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). In the former case, slides were digitalized and printed using a Polaroid SprintScan 4000 film scanner (Polaroid Corp., Cambridge MA) run on Microsoft Windows 98. In the latter case, microscopic fields were digitalized thanks to a software specifically designed for image acquisition and storage (Leica IM 50). Image adjustment and printing were achieved with appropriate softwares (Corel PHOTO-PAINT™ and CorelDRAW™, Corel Corporation, Ottawa, ON Canada).

Immunochemical and biochemical methods

Western blot analysis. IBEP-2 cells in Phenol Red-free RPMI 1640 (Gibco™, Invitrogen, Merelbeke, Belgium) supplemented with 10% charcoal-stripped FBS were plated in 60-cm^2 Petri dishes (density 10^6 cells/dish). Two days after seeding, cells were fed fresh RPMI 1640 with charcoal-stripped FBS, containing estrogen or estrogen antagonists at the concentrations indicated in 'Results'. After 5 or 24 h of incubation, cell lysates were prepared directly from monolayer cultures. All subsequent steps were run at 4°C using ice cold buffers. Culture medium was removed and cells were rinsed twice with TBS (50 mM Tris–HCl pH 7.5 and 150 mM NaCl). Cell lysis was achieved *in situ* using 0.5 ml of a lysis solution (TBS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 0.1 mM Na_3VO_4 and 5 mM EDTA) with freshly added proteolysis inhibitors (0.6 mM PMSF and 0.3 mM TPCK). Dishes

were left with this solution at 4°C for 15 min and cell monolayers were then scraped. Resulting lysates were harvested, transferred into microfuge tubes and then passed three times through a 1 ml syringe fitted with a 0.4 mm × 19 mm needle. After 15 min of further incubation, lysates were clarified by centrifugation at 17,000*g* (30 min at 4°C). Supernatants (total cell extracts) were distributed in 100 µl aliquots and stored at −80°C until biochemical analysis. Total cell lysates were mixed with loading buffer (220 mM Tris–HCl pH 6.7, 17.5% glycerol, 8.5% β-mercaptoethanol and 5% SDS) to obtain a final protein concentration of 0.5 mg/ml. Denatured samples (25 µg protein) were subjected to 10% SDS-PAGE and subsequently electrotransferred onto nitrocellulose membranes (Amersham Belgium, Gent) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Nazareth Eke, Belgium). The membranes were then incubated for 3 h at room temperature in a blotting solution (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20 and 7% skim milk) to prevent non-specific binding. In order to assess the expression of ER, immunoblotting was performed with the same anti-ER antibodies as those indicated above (H-184, HC-20 from Santa Cruz Biotechnology). The membranes were incubated overnight at 4°C in a fresh blotting solution containing diluted antibody (H-184, 1:2,000 dilution or HC-20, 1:10,000 dilution). Immunoblots were thoroughly washed (blotting solution without milk) and then incubated for 2 h at room temperature in the blotting solution containing peroxidase-labeled donkey anti-rabbit IgG antibody (1:10,000) (Amersham Belgium, Gent). Finally, after extensively washing (blotting solution without milk), bound peroxidase activity was revealed using the Lumi-Light Western Blotting Substrate from Roche Diagnostics Belgium (Brussels). A similar procedure, with minor modifications, was applied for PgR demonstration. In the latter case, samples containing 50 µg protein were resolved by 8% SDS-PAGE. The primary reagent was a mixture of two monoclonal antibodies raised against A and B isoforms of PgR (NCL-PGR-AB, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), used at 1:250 dilution. Bound primary antibodies were detected with a peroxidase-labeled sheep anti-mouse IgG antibody (Amersham Belgium, Gent) at 1:5,000 dilution. The relative density of immunoreactive bands was estimated using a computer-assisted gel scanning densitometer (GS-710 Calibrated Imaging Densitometer) and Quantity One software, both from Bio-Rad (Hercules, CA).

Measurement of ERs and PgRs by enzyme-linked immunoassay (EIA). Expression of ER and induction of PgR upon treatment with estrogen agonist and antagonists were investigated by EIAs with kits from Abbott Diagnostics (Louvain-la-Neuve, Belgium). IBEP-2 cells or MCF-7 cells in Phenol Red-free RPMI 1640 supplemented with 10% charcoal-stripped FBS were plated in 75-cm^2 flasks (density 10^4 cells/cm^2). Two days after plating, cells were cultured for 24 h in fresh medium containing E_2 or estrogen antagonists at the concentrations indicated in 'Results'. After treatment, cells were gently rinsed twice with 10 ml of Ca^{++}, Mg^{++}-free Hank's balanced salt solution (HBSS) and dislodged from the substratum with 1 mM EDTA in HBSS at 37°C. All further steps were performed at 4°C using ice cold buffers. Cell suspensions were collected in 15-ml conic tubes and the flasks were rinsed three times with 3 ml of Ca^{++}, Mg^{++}-free HBSS. The resulting suspensions were centrifuged at 250*g* for 10 min. The pellets were washed twice with 10 ml of Ca^{++}, Mg^{++}-free HBSS and finally suspended in 0.4 ml of 10 mM phosphate buffer pH 7.4 containing 10% glycerol, 1.5 mM EDTA, 1 mM β-mercaptoethanol and 0.5 M KCl. Total cellular extracts were obtained by three freeze-thawing cycles of the cell suspensions and subsequent clarification by ultracentrifugation for 30 min at 100,000*g*. ER and PgR concentrations in KCl extracts were measured using the corresponding EIA kits, according to the manufacturer's recommendations. Experiments were performed in duplicate. Concentrations were expressed in fmol per mg of protein.

Protein assay. Protein concentrations in total cell lysates obtained by detergent extraction (see Western blot analysis) were determined by the bicinchoninic acid method [20] using the BCA™ Protein Assay Reagent from Pierce (Rockford, IL). Proteins in KCl extracts (see EIA) were assayed by the method of Bradford [21] using the Coomassie® Protein Reagent Assay from the same manufacturer. In both cases, reference curves were constructed with bovine serum albumin.

Results

In order to compare the levels of ER expression in IBEP-2 and MCF-7 cell lines, concentrations of ER protein were determined by EIA in cells cultured in absence of estrogenic stimulation. The value

obtained for IBEP-2 (662 ± 18 fmol/mg cell protein) appeared slightly higher than that found for MCF-7 (595 ± 37 fmol/mg cell protein, mean of four determinations ± SD). Thus, both cell lines did not differ markedly with respect to ER expression. Of note, the value of ER level that we determined in MCF-7 cells was in the same range as that reported previously by others (413 fmol/mg cell protein) [22].

The presence of ER in IBEP-2 cell line can be expected to result in a sensitivity to estrogenic stimulation. The proliferative response of IBEP-2 and MCF-7 cells to the presence in culture medium of agonists endowed with different estrogenic potencies is illustrated in Figure 1. Both cell lines typically exhibited sigmoid dose–response curves, with a maximum response reaching 3.5-fold baseline value. According to the decreasing estrogenicity of the different agonists ($E_2 \approx$ DES > E_1 > genistein), EC_{50}'s characterizing IBEP-2 response ranged from 2.4×10^{-12} M (for E_2) to 1.8×10^{-8} M (for genistein). Based on EC_{50} values, IBEP-2 appeared slightly more sensitive than MCF-7 to E_2 (2.4×10^{-12} M v.s. 10^{-11} M), E_1 (4.2×10^{-11} M v.s. 8.7×10^{-11} M) and genistein (although in the latter case the difference was small, that is, 1.8×10^{-8} M v.s. 2.7×10^{-8} M). By contrast, both cell lines displayed similar sensitivity to DES.

Pure (steroidal) estrogen antagonists, as well as partial antiestrogens, compete with estrogens for the receptor and suppress the effects of estrogenic stimulation. As shown in Figure 2, addition of increasing concentrations of ICI 182,780 or OH-Tam to IBEP-2 cells growing in presence of 10^{-10} M E_2 caused

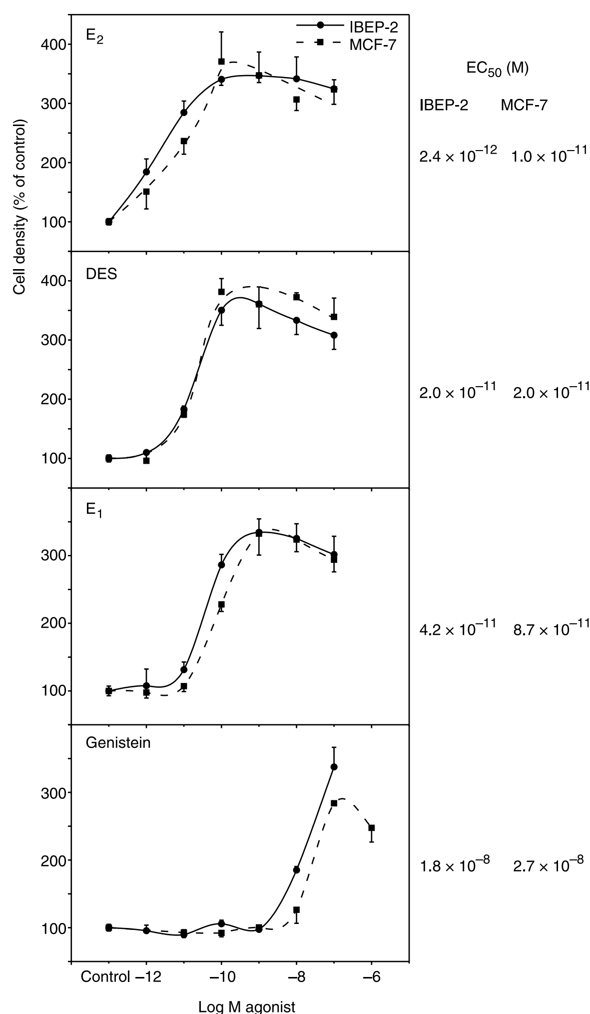

Figure 1. Proliferative response of IBEP-2 and MCF-7 cell lines to different estrogen agonists. Cell densities were measured after three days of exposure to estrogenic compounds, as described in 'Methods'. Control, cells cultured in absence of estrogenic stimulation. Each symbol is the mean of four determinations ± S.D.

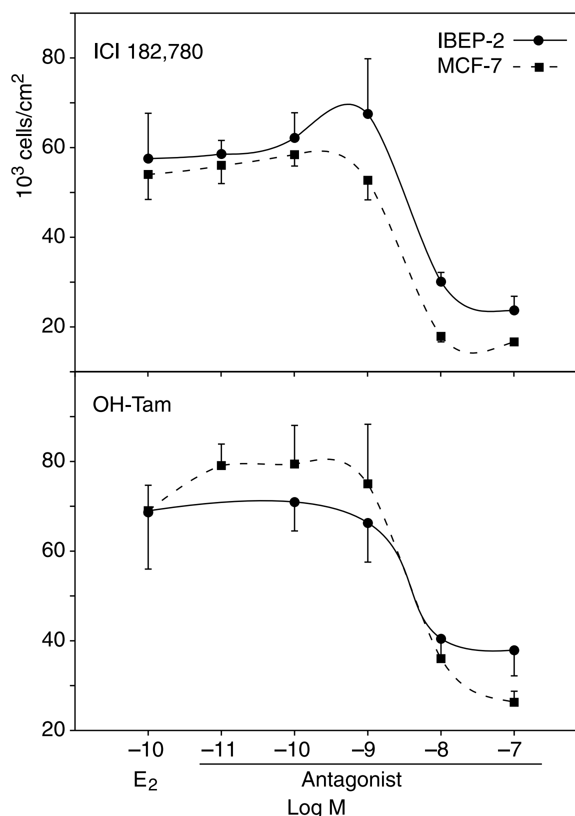

Figure 2. Inhibition of IBEP-2 and MCF-7 cell growth by antiestrogens ICI 182,780 and OH-Tam. Cell densities were measured after 3 days of culture in presence of 10^{-10} M 17β-estradiol without or with antiestrogens at increasing concentrations. Each symbol is the mean of four determinations ± S.D.

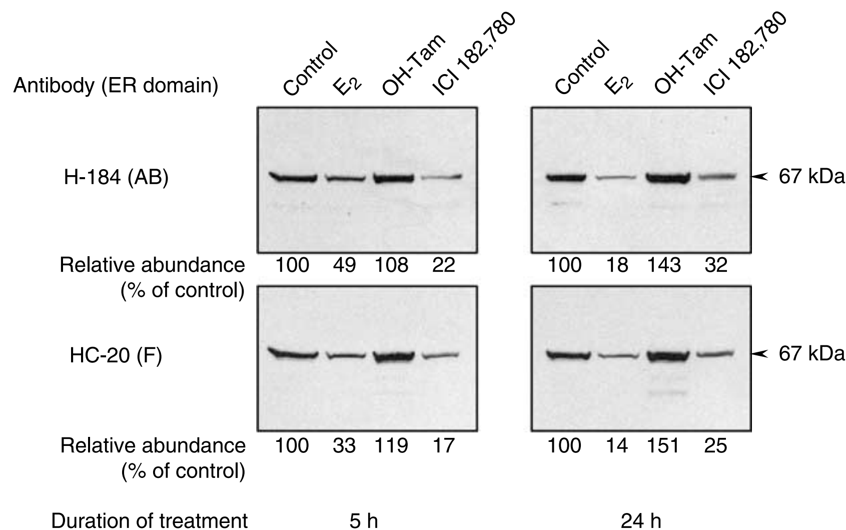

Figure 3. Estrogen- and antiestrogen-induced modulation of ER expression in IBEP-2 cells, revealed by SDS-PAGE and immunoblotting using antisera raised against peptide sequences in the aminoterminal, AB domain (H-184) or the carboxyterminal, F domain (HC-20). Cells were processed for ER analysis after 5 or 24 h of exposure to either 10^{-9} M E_2 or 10^{-7} M estrogen antagonist. Control, no drug. The relative abundance of immunoreactive protein was estimated by computer-assisted densitometry, using as a reference (100%) the density of ER band in untreated cells.

a gradual decrease of cell proliferation. Both antiestrogens displayed similar inhibitory potencies, with IC_{50}'s around 3×10^{-9} M. IC_{50}'s of antiestrogens on MCF-7 cells were not markedly different although the latter cells appeared slightly more sensitive to high drug concentrations (10^{-8}–10^{-7} M) (Figure 2).

Ligand binding is known to modulate ER level by altering mRNA transcription/stability and the turnover of the protein. The effects of E_2 and estrogen antagonists on ER content in IBEP-2 cells were examined by Western blot analysis and by immunofluorescence, using two different antisera (H-184 and HC-20) raised against distinct regions of ER protein. Results of ER assessment by immunoblotting are presented in Figure 3. Cell exposure to E_2 for periods of 5 or 24 h produced a substantial decrease of the band associated with immunoreactive ER. A similar reduction of immunoreactive ER was seen in cells exposed to ICI 182,780. By contrast, the intensity of the band reflecting the amount of immunoreactive ER was not diminished, but rather increased after exposure to OH-Tam. It is noteworthy that identical results were obtained previously with MCF-7 cells exposed to OH-Tam or the steroidal antiestrogen RU 58,668 [23].

Close examination of Figure 3 (compare left and right panels) also reveals that E_2 caused in IBEP-2 cells a progressive loss of immunoreactive ER while ICI 182,780 only induced a transient decrease followed by a partial restoration of ER level. Interestingly, comparable results were obtained in a previous study of Jensen et al. [24] examining ligand-induced ER modulation in MCF-7 cells. Such a difference in effect between E_2 and ICI 182,780 seems to be due to the fact that the former produces a rapid (i.e., within hours) and sustained downregulation of ER mRNA, whereas exposure to the latter results in a delayed (i.e., after 24 h) ER mRNA upregulation [24].

ER content in IBEP-2 cells treated with E_2 and the same estrogen antagonists was also examined by immunofluorescence. Results are shown in Figure 4. In absence of estrogenic stimulation, ER immunostaining produced a strong signal essentially associated with cell nuclei, whatever antiserum was used in the immunocytochemical procedure (Figures 4(a and b)). Exposure to E_2 or ICI 182,780 led to a drastic decrease of the nuclear signal associated with immunoreactive ER (Figures 4(c, d and g, h). Interestingly enough, this change was less apparent with the use of antiserum H-184 than with HC-20 (compare Figures 4(c, g and d, h). On the other hand, the effect of OH-Tam on ER expression was radically different since this antagonist induced an intensification rather than an attenuation of ER immunostaining (Figures 4(e and f)). As illustrated in Figure 5, ER decrease induced in IBEP-2 cells by E_2 or ICI 182,780 (Figures 5(a, c and e)) involved protein degradation in the proteasomes, since it was totally (E_2, Figure 5(d)) or partially (ICI 182,780, Figure 5(f)) prevented by the proteasome

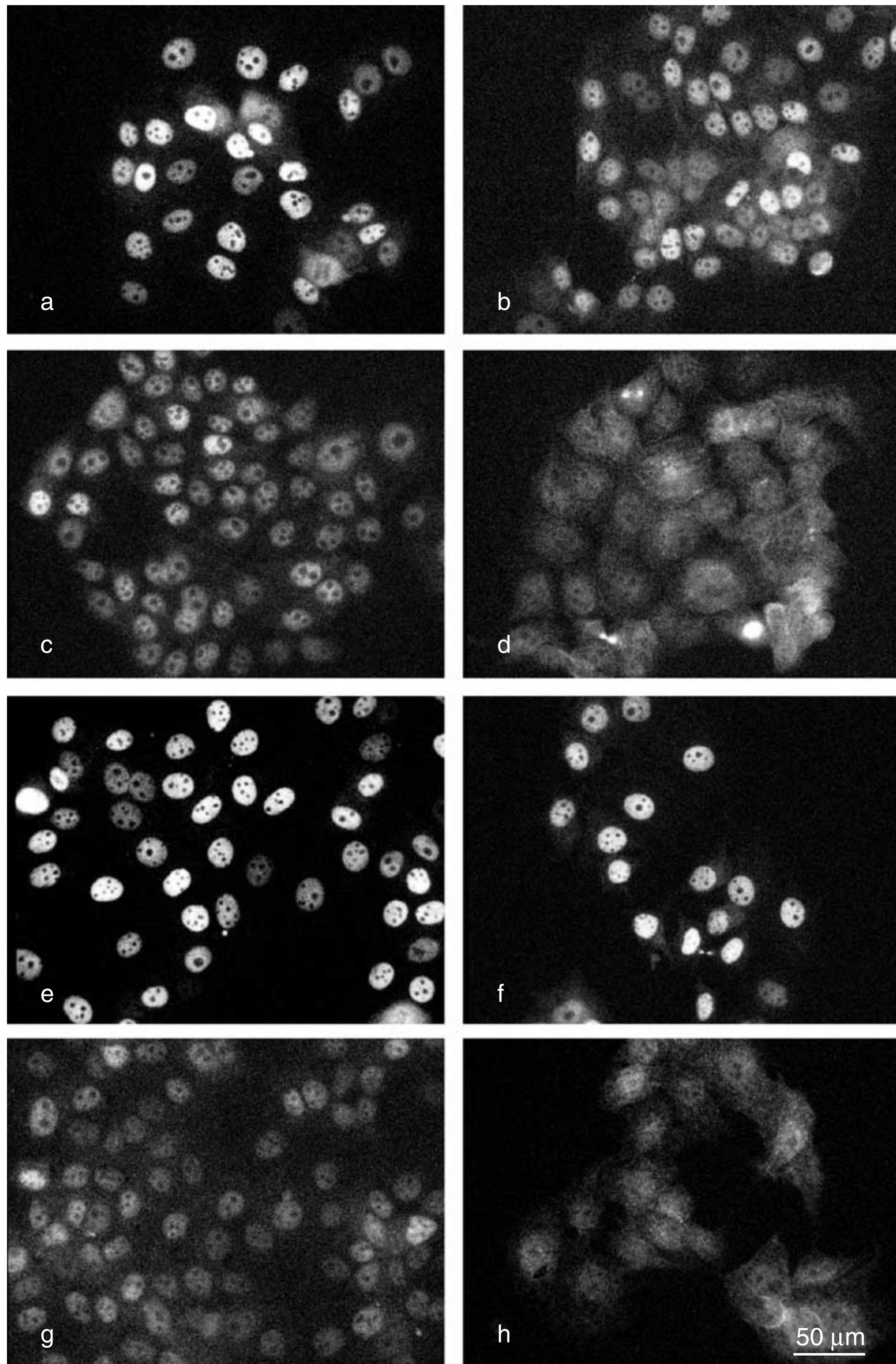

Figure 4. Estrogen- and antiestrogen-induced modulation of ER expression in IBEP-2 cells, as demonstrated by immunofluorescence using as primary antisera the same as those in Figure 3 (a, c, e, g: H-184; b, d, f, h: HC-20). a, b: no drug; c, d: 10^{-9} M E_2; e, f: 10^{-7} M OH-Tam; g, h: 10^{-7} M ICI 182,780 (25 h exposure). Texas Red labeling.

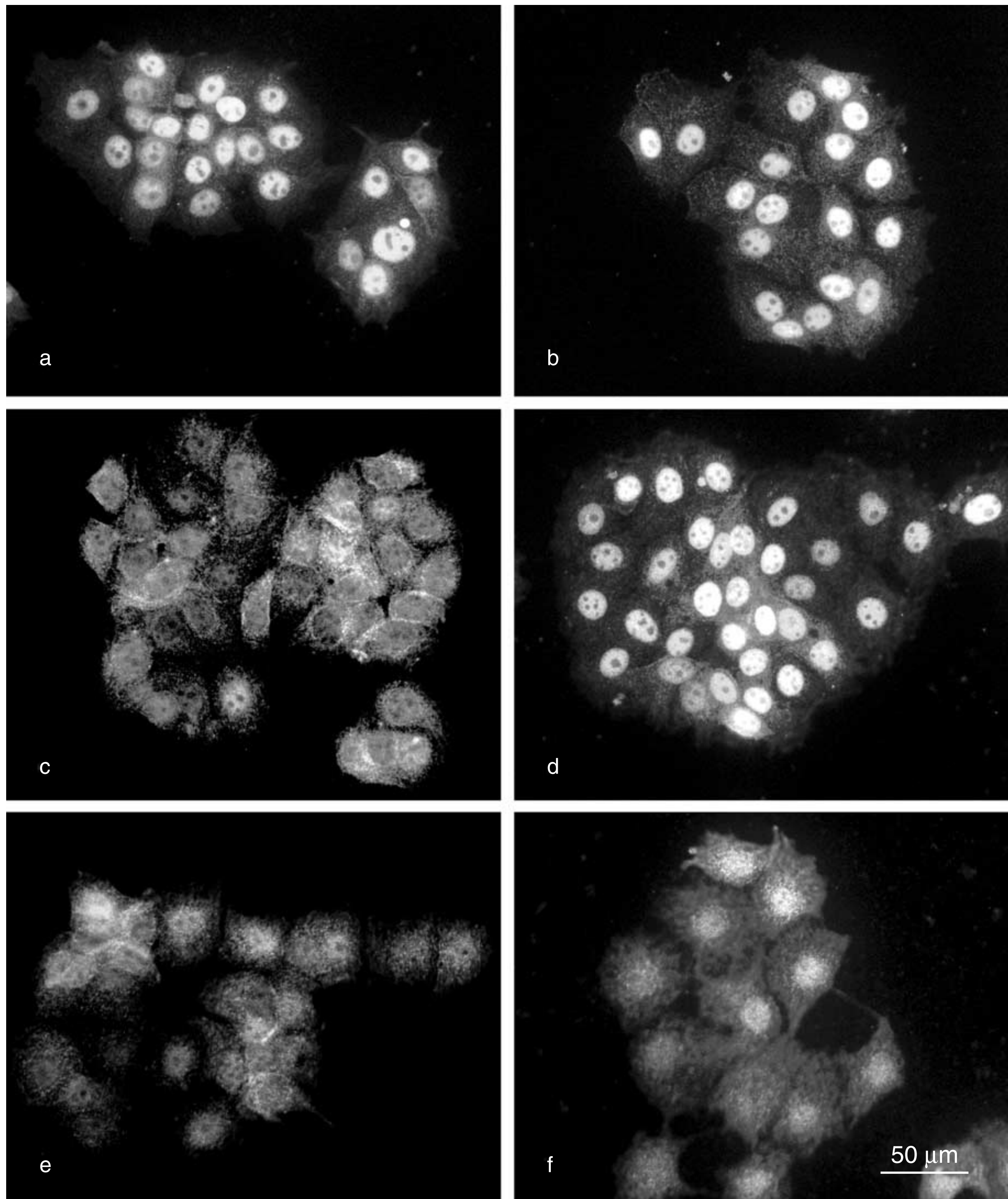

Figure 5. Effect of MG-132 on ER downregulation induced in IBEP-2 cells by E_2 or ICI 182,780. Immunofluorescence was performed with HC-20 antiserum. a: no drug, b: 10^{-5} M MG-132 (6 h exposure); c, d: 10^{-9} M E_2 alone (c) or in combination with 10^{-5} M MG-132 (d); e, f: 10^{-7} M ICI 182,780 alone (e) or in combination with MG-132 (f). Exposure to E_2 or ICI 182,780 (5 h) was started 1 h after addition of MG-132. Texas Red labeling.

Table 1. Levels of PgRs in IBEP-2 and MCF-7 cells[a]

Cell line	Baseline value[b]	E_2[c]	E_2 + OH-Tam	E_2 + ICI 182,780
IBEP-2	12.6 ± 3.8[d]	321.4 ± 41.5	126.8 ± 4.0	11.9 ± 3.6
MCF-7	38.0 ± 1.9	621.4 ± 26.5	n.d.[e]	n.d.

[a] Determined by EIA, as detailed in 'Methods'.
[b] PgR level in cells grown for at least 24 h in absence of estrogenic stimulation.
[c] 24-h exposure to 10^{-9} M E_2 alone, or with 10^{-7} M antiestrogen.
[d] fmol/mg protein, mean of four determinations ± S.D.
[e] Not determined.

inhibitor MG-132. Expectedly, addition of MG-132 alone resulted in the same strong nuclear signal as that observed in untreated cells (Figure 5(b)).

Mitogenic response of sensitive cells to estrogens, as seen above, is the end result of ER activation and action at the genomic level. Induction of ER transcriptional activity by estrogens can also been revealed by examining the expression of specific target genes such as the gene of the PgR. The effect of estrogenic stimulation on PgR content in IBEP-2 and MCF-7 cells was first evaluated by EIA (Table 1). Exposure to E_2 resulted in a 25-fold increase of PgR in IBEP-2 cells, indicating a dramatic effect of E_2 on PgR expression. It is noteworthy that the level of PgR protein measured by EIA in E_2-treated IBEP-2 cells corresponded to the value of binding sites determined previously in these cells by Scatchard analysis [11]. As could be expected, estrogen-induced PgR expression was sensitive to antiestrogens although in this respect ICI 182,780 exerted a stronger inhibitory effect than OH-Tam at the same concentration. Incidentally, the baseline level of PgR in MCF-7 cells was approximately three times higher, compared with IBEP-2 cells. Furthermore, the augmentation of PgR expression due to E_2 was less pronounced in MCF-7 (16-fold) than in IBEP-2, even though in presence of E_2 PgR content in MCF-7 cells was twice that noted in IBEP-2 cells.

Human PgR occurs as two isoforms, the full length isoform B (PgR-B, 933 residues, 120 kDa) and the N-terminally truncated isoform A (PgR-A, 769 residues, 94 kDa) which are transcribed from distinct promoters within a single copy of the PgR gene. In most human as well as other mammalian PgR-expressing cells, B and A isoforms act in opposite ways, the former behaving as a transcriptional activator and the latter functioning as a transcriptional inhibitor of other steroid receptors (including ER) [25]. Furthermore, microarray analysis of gene expression in breast carcinoma cell lines engineered to express one or the other PgR isoform clearly shows that PgR-B and PgR-A exert

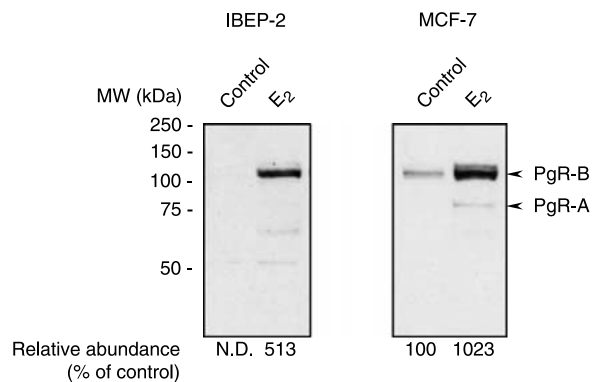

Figure 6. Estrogen induction of PgR expression in IBEP-2 and MCF-7 cells, revealed by SDS-PAGE and immunoblotting using monoclonal antibodies raised against both A and B isoforms of the protein (see Methods). Cells were processed for PgR analysis after 24 h of exposure to 10^{-9} M E_2. Control, no drug. The relative abundance of immunoreactive protein was estimated by computer-assisted densitometry, using as a reference (100%) the density of PgR-B band in MCF-7 cells.

their control on different subsets of genes, even though some genes can be regulated by both receptor isoforms [26]. In the current study, Western blot analysis was used to distinguish PgR isoforms in IBEP-2 and MCF-7 cells exposed to estrogenic stimulation. As shown in Figure 6, the major PgR isoform induced by E_2 exposure in both IBEP-2 and MCF-7 cells was the B one. In these conditions, IBEP-2 cells expressed no detectable A isoform, and only a small amount of the latter was detected in MCF-7 cells.

PgR content in IBEP-2 cells was also assessed by immunofluorescence using an antiserum raised against both isoforms of the PgR protein. As illustrated in Figure 7(a), cells deprived of estrogen exhibited a weak fluorescence mainly at the nuclear level. The presence of E_2 in the culture medium resulted in a substantial increase of fluorescence in the nuclei, reflecting the augmentation of PgR expression (Figure 7(c)). Of note, a similar pattern (signal intensity and distribution) was observed when the fluorochrome FITC was

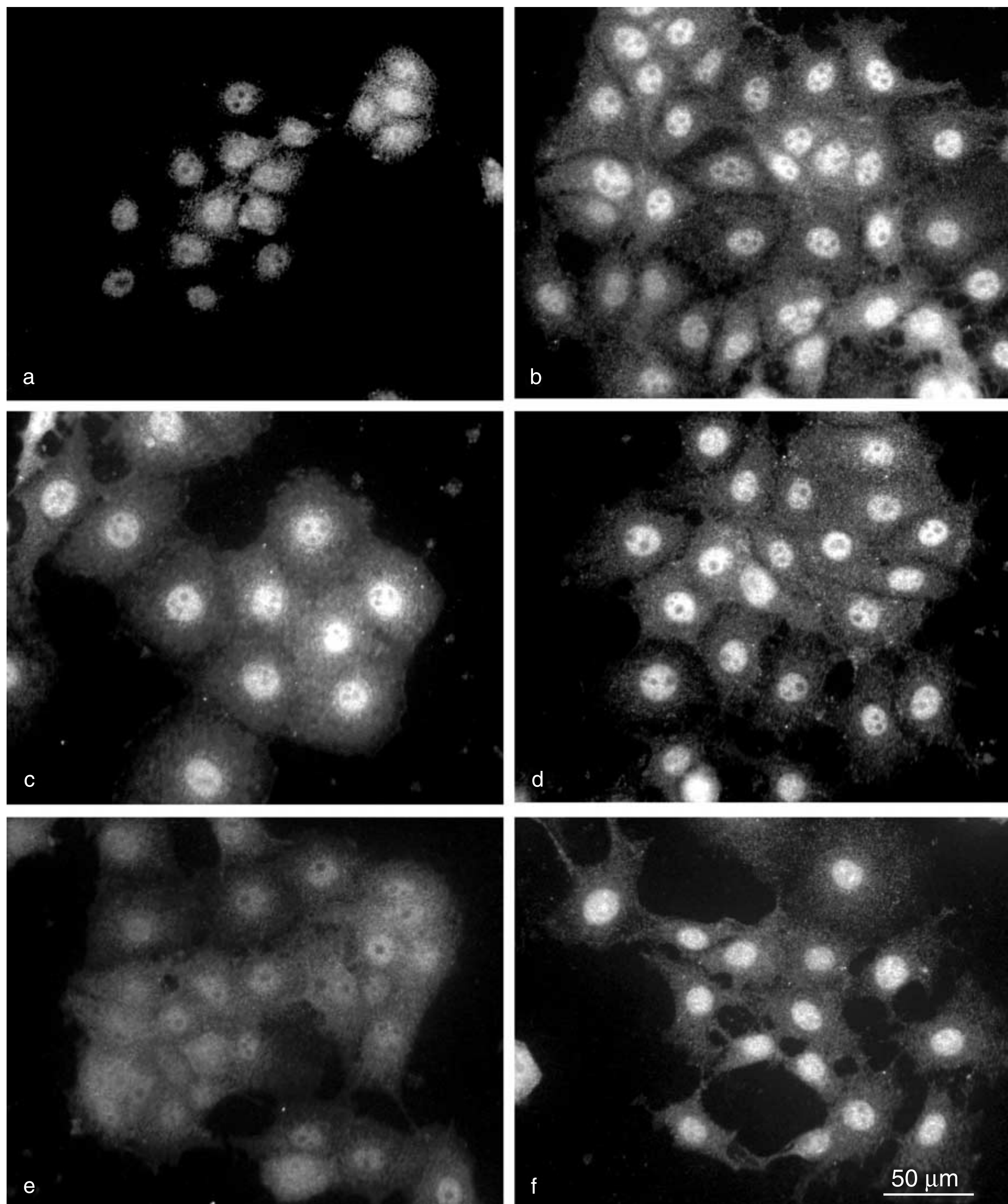

Figure 7. Effect of E_2 and MG-132 on PgR expression in IBEP-2 cells, demonstrated by immunofluorescence using C-19 antiserum raised against both A and B isoforms of PgR. a: no drug; b: 10^{-5} M MG-132; c, d: 10^{-9} M E_2 alone (c) or in combination with 10^{-5} M MG-132 (d); e, f: 10^{-7} M ICI 182,780 alone (e) or in combination with MG-132 (f). Duration of drug exposure as indicated in legend to Figure 5, cells fixed 18 h after cessation of drug treatment. Texas Red labeling.

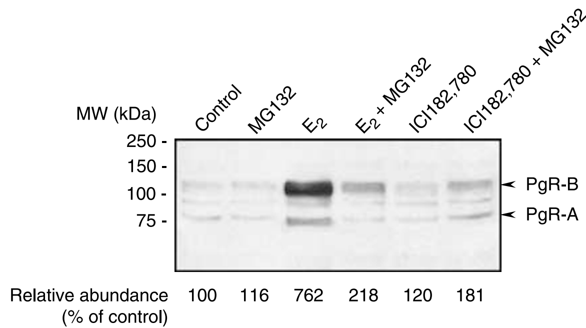

Figure 8. Effect of MG-132 on E_2-induced augmentation of PgR expression in IBEP-2 cells, as demonstrated by SDS-PAGE and immunoblotting. Duration of treatment as indicated in legend to Figure 5, except that cells were processed for analysis 18 h after cessation of treatment. The relative abundance of immunoreactive protein was estimated as indicated in legend to Figure 6, except that untreated IBEP-2 cells were used as a reference (100%). Note that the duration of film exposure was increased in order to visualize PgR-B immunoreactive band in untreated cells.

used instead of Texas Red for immunolabeling (data not shown).

So as to explore a possible relationship between agonist-induced ER downregulation and the induction of PgR, IBEP-2 cells exposed to E_2 alone or in combination with MG-132 were processed for PgR immunostaining. Results are illustrated in Figures 7(b–d). Inclusion of MG-132 in the system did not completely suppress PgR induction by E_2 (compare Figures 7(c and d) and, amazingly, MG-132 alone increased the signal associated with immunoreactive PgR (Figure 7(b)). That augmentation of PgR due to MG-132 was estrogen-independent since it was not antagonized by ICI 182,780 (Figures 7(e and f)). Morphological observations based on immunofluorescence staining were largely corroborated by Western blot analysis (Figure 8), even though the latter approach barely revealed PgR increase induced by MG-132 alone. Thus, MG-132 diminished but did not abolish E_2-induced PgR expression. Furthermore, a moderate increase of PgR content was seen in cells treated with the proteasome inhibitor despite the concomitant presence of ICI 182,780.

Discussion

The purposes of the current study were to assess ER and PgR, evaluate receptor function (ER-induced PgR expression) and examine ligand-mediated modulation of ER expression in the recently established cell line IBEP-2.

Two types of ERs have been identified (ERα and ERβ). They share similarities in structure but differ in tissue distribution and mode of action [27]. ERβ seems to be the most prominent isoform expressed in normal mammary gland [28], whereas both receptors have been reported in breast cancer cells [29, 30]. On the other hand, ERα is the dominant subtype in MCF-7 cells and a possible production of ERβ by these cells still remains a matter of debate, since reports are conflicting [29, 31, 32]. Inasmuch as the antisera that we used for immunodetection were not cross-reactive with ERβ (at least according to the supplier's specifications), our data most probably reflect the expression and behavior of ERα. Likewise, we assume that the proliferative response of MCF-7 cells to agonists is due to ERα, even though we cannot exclude the possibility of ERβ involvement in IBEP-2 cells.

As demonstrated previously by various assay methods [33–35] estrogen agonists typically induce an increase of proliferative activity in MCF-7 cells. Occasional reports [e.g., 36] of a lack of mitogenic effect of estrogens on MCF-7 cells presumably result from inappropriate methodology. Even though the EC_{50}'s that we inferred from the response curves to various estrogen agonists somehow differed in absolute value from previously published data [35], we obtained for both IBEP-2 and MCF-7 a similar ranking of compounds on the basis of mitogenic potencies ($E_2 \approx$ DES > E_1 > genistein). Besides, IBEP-2 cells seemed to be more sensitive to steroidal estrogens E_2 and E_1 than MCF-7, whereas no or minimal difference was found when stilbene (DES) and isoflavonoid (genistein) compounds were tested. As revealed by EIA, the higher sensitivity of IBEP-2 cells to the mitogenic effect of E_2 and E_1 was not matched by a major difference in ER levels. Furthermore, previous estimates show that the binding capacity of ER in IBEP-2 is approximately two-fifth less than in MCF-7 [11, 23]. Since the difference in sensitivity varied according to the agonist, it cannot be explained by cell-related variation in cofactor recruitment by receptor–ligand complexes. It could possibly be related to cell-dependent difference in metabolism, resulting in a more or less rapid inactivation of agonist mitogenicity.

Whereas IBEP-2 and MCF-7 cell lines differed in their proliferative response to agonists, they seemed to be equally sensitive to antiestrogens. Of note, the IC_{50} value that we determined for the inhibitory activity of OH-Tam on MCF-7 was similar to that reported in a previous study [37]. Unlike others [34], we did not find any difference in potency between ICI 782,780

and OH-Tam. This could result from variation in methodology, namely the shorter duration of antiestrogen exposure in our proliferation assay. Indeed, increasing length of treatment with antiestrogens not only impairs cell proliferation but also induces cell apoptosis and, in this respect, ICI 182,780 is more effective in causing cell death as compared to OH-Tam [38].

In IBEP-2 cells, as well as in MCF-7 cells, gene transactivation mediated by liganded ER resulted in the induction of PgR. The increase of PgR expression upon treatment with E_2 seemed to preferentially affect the B isoform (PgR-B). Previous work by others also reports PgR-B induction by E_2 in MCF-7 cells [39]. On the other hand, our observations are somehow at variance with a recent study of Vienonen et al. [40] reporting a stronger induction of PgR-A as compared to PgR-B in MCF-7 cells exposed to estrogenic stimulation. The reason for this apparent discrepancy remains elusive. It cannot be attributed to the nature of the primary antibody (in our hands, the same antibody as that used by Vienonen did not demonstrate preferential induction of PgR-A in IBEP-2 cells). A possible explanation might be found in differences in cell culture conditions, namely the use of insulin supplementation in Vienonen's work. Insulin might indeed modulate E_2-mediated PgR induction by acting via the IGF-1 receptor [41]. In this context, it is noteworthy that the magnitude of E_2-induced PgR expression reported in Vienonen's work (4–5×) is considerably less than that observed in our study.

In a physiological point of view, PgR-B is the most important PgR isoform for the proliferation and differentiation of normal mammary epithelium since studies on knock-out mice have shown that normal breast development occurs in absence of PgR-A expression [42]. Studies published in the recent literature indicate that the involvement of PgR isoforms in breast cancer could be quite complex. As revealed by the analysis of PgR expression in a large number of human breast neoplasms, tumor cells generally produce both PgR isoforms, even though there is frequently a predominance of PgR-A over PgR-B due to reduced expression of the latter [43]. Excess of PgR-A over PgR-B is also associated with a more undifferentiated tumor phenotype [44]. The fact that we found a predominant expression of PgR-B in IBEP-2 and MCF-7 cells under conditions of estrogenic stimulation suggests that these cell lines derived from atypical cases of breast cancers with an elevated PgR-B expression (and almost no PgR-A expression). Another possibility would be that a PgR-B predominance over PgR-A could develop during tumor cell adaptation to growth *in vitro*.

An elegant model developed by Horwitz et al. has enabled one to obtain a further insight into the impact of PgR-A and PgR-B on the phenotype of breast carcinoma cells [45, 46]. This model relied on the transfection of PgR-negative T47D cells (T47D-Y) with either PgR-A (T47D-YA) or PgR-B (T47D-YB) expression vectors in order to generate sublines expressing only one isoform. This gave the opportunity of examining the effect of PgR-A or PgR-B on tumor growth in vivo by injecting cells to nude mice. Surprisingly, in this model PgR-A-expressing tumors grew more slowly than PgR-B-expressing neoplasms, indicating a negative effect of the former isoform on tumor development [46]. This suggests that in human breast carcinoma PgR-A expression is not the only element involved in more aggressive growth.

As revealed by a number of studies on breast cancer cells and other ER-positive cell lines, ER expression is modulated by ligand binding through complex regulation mechanisms acting at transcriptional, posttranscriptional and posttranslational levels, and governing receptor synthesis and degradation. From previous studies [47] it is known that ER regulation upon ligand binding can follow distinct pathways, depending on the cell line. In the MCF-7 cell line, estrogens cause a decrease of ER mRNA and protein (downregulation, model I regulation). Conversely, exposure of T47D cell line to estrogen induces an increase of ER mRNA level and produces only a transient decline of ER protein level (model II regulation). Furthermore, ligand-induced modulation of ER content in a given cell line also depends on ligand structure. While in MCF-7 and T47D cells, steroidal antiestrogens induce ER downregulation, treatment with triphenylethylene-based antagonists (tamoxifen) leads to ER accumulation (upregulation) [47–50]. The current study shows that this is also the case for IBEP-2 cells.

Obviously, estrogen-induced ER regulation in IBEP-2 cell line produced a decrease of receptor protein and thus occurred according to model I. As mentioned above, ER downregulation involves both a fall of mRNA level [51] and a rapid degradation of the protein. Although some observations suggest that ER proteolysis could take place in the lysosomal compartment [52], compelling evidence in the recent literature points to a major involvement of proteasomes [12–18]. In accordance with other studies, our observations show that the proteasome inhibitor MG-132

suppresses ligand-mediated downregulation of ER in IBEP-2.

Regulation of receptor level by proteasome-mediated proteolysis is by no means specific to ER since it has also been observed for other nuclear receptors, including PgR [53], the thyroid hormone receptor [54], the retinoid X receptor [55], the glucocorticoid receptor [56] and the aryl hydrocarbon receptor (AhR) [57]. In the latter case, ligand binding induces not only the proteasome-dependent degradation of the cognate receptors (i.e., AhR), but also that of ER by a still undefined cross-talk mechanism [57]. Ligand-induced degradation of nuclear receptors probably contributes to the control of cell response. However the relationship between receptor degradation and receptor-mediated transactivation is not straightforward and remains partially unraveled. Indeed, it seems that the impact of receptor proteolysis on transactivation vary according to the nature of the receptor, the cellular context and/or the target gene (cellular or reporter gene). Whereas an impairment of ER degradation in MCF-7 cells has been reported to decrease transactivation [15], inhibition of glucocorticoid receptor downregulation in transfected T47D cells enhances receptor-mediated transactivation [56]. Furthermore, in pituitary lactotrope cells suppression of E_2-induced ER downregulation by thyroid hormone does not interfere with ER-mediated induction of prolactin gene expression [58].

In contrast with what was found previously for MCF-7 cells [15], treatment of IBEP-2 cells with the proteasome inhibitor MG-132 did not abolish E_2-induced induction of PgR. However, the interpretation of this finding is not straightforward, insofar as MG-132 by itself produced an estrogen-independent accumulation of PgR protein. Thus, we cannot exclude that a possible attenuation of PgR induction by proteasome inhibition could be counterbalanced by an increase of PgR protein due to altered turnover. In other words, in our experimental conditions the effect of MG-132 on PgR downregulation might obscure functional consequences of altered ER regulation. Anyway, it remains intriguing that inhibition of PgR disposal in proteasomes could lead to such an accumulation in a matter of few hours, considering the relatively long half-life (25 h) which has been reported for this receptor in MCF-7 cells [59]. More recent data suggest however that, in other cell lines, PgR turnover might be more rapid than originally thought [45].

To conclude, we show in the current study that IBEP-2 cell line resembles MCF-7 in many aspects but exhibits slightly higher sensitivity to steroidal estrogens and stronger induction of PgR expression in response to E_2. By showing a similarity of behavior between the recently established cell line IBEP-2 and MCF-7 which was derived 30 years ago [7], our observations strengthen the validity of cell culture approaches for the study of human breast carcinoma. Besides, the marked expression of PgR when IBEP-2 cell line is exposed to estrogenic stimulation makes this cell line particularly valuable as an experimental model to study the modalities of estrogen-mediated PgR induction.

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