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Preclinical studies

Interaction between estrogen receptor alpha, ionizing radiation and (anti-) estrogens in breast cancer cells

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Key words: breast cancer, estrogen receptor, ionizing radiation, *in vitro*

Summary

Purpose. Estrogen receptor alpha (ERα) plays a major role in breast cancer development. It acts as ligand-inducible transcription factor which determines growth, survival and differentiation of breast cancer cells. The aim of this study is to evaluate the potential interference between radiotherapy and estrogen receptor responsiveness.

Materials and methods. The effect of ionizing radiation was assessed on the estrogen receptor alpha status, growth (proliferation and apoptosis) and sensitivity of MCF-7 breast cancer cells to estrogenic (17β-estradiol (E_2)), selective estrogen receptor modulator (SERM) and anti-estrogenic compounds.

Results. We have observed a ligand-independent decrease in ERα expression after radiation, resulting from a specific reduction in mRNA level and protein synthesis. This ERα disappearance occurred 72 h post-irradiation at 8 Gy and decreased the transcriptional activity in ERα of these cells. On the other hand, E_2 impedes the growth inhibitory effects (essentially on proliferation) of ionizing radiation in MCF-7 cells, which potentially decreases radiosensitivity of these cells. This effect was totally blocked by SERM and anti-estrogenic treatments. Moreover, this growth effect of concurrent anti-estrogenic drugs and ionizing radiation appeared to be strongly synergistic

Conclusions. This study may increase general comprehension of ERα modulation by radiotherapy and improve adjuvant therapeutic approaches based on co-administration of radiation and endocrine therapy.

Introduction

Breast cancer is the most common cancer affecting women in Europe and the USA: 12.5% of women will develop breast cancer during their life [1]. More than 300,000 new cases are diagnosed every year in Europe [2]. According to the American Cancer Society, 216,000 new cases should be observed in 2004 in the USA (www.cancer.org), with an incidence increasing steadily.

A majority (70%) of breast tumors express the alpha subtype of the estrogen receptor (ERα) [3] and ERα status is used as a predictive factor for hormone therapy. ERα-positive tumors have a 60–80% overall response rate to the SERMs (i.e. tamoxifen) [4]. The presence of ERα is also associated with increased survival and longer disease free intervals.

Primary treatment is normally breast-conserving surgery or mastectomy, in association with axillary's surgery and radiation therapy. Numerous prospective and retrospective trials demonstrated that a breast conserving surgery, consisting of segmental mastectomy with or without axillary's lymph node dissection, is equivalent to mastectomy in terms of overall and disease free survivals for patients with early stage breast carcinoma. Radiotherapy prevents local recurrence of breast cancer after breast-conserving surgery [5–7]. Both hormone therapy and adjuvant radiotherapy remain major treatments to manage early breast cancer [4]. Recently, the clinical advantage of concomitant hormonotherapy/radiotherapy in terms of efficacy has been suggested [8].

Until now, no rational clinical attitude has been proposed regarding the combination of hormone and adjuvant therapy. Biological explanation from *in vitro* studies do not provide any suggestion due to the large variety of investigated experimental conditions [9]. The potential impact of radiation on the ERα level and associated transcriptional activity is not established. In this regard, we reported in a previous study a decreased of estrogen binding ability in MCF-7 breast cancer cells irradiated at time of plating [10]. In the present paper, we further analyzed the impact of ionizing radiation on estrogen receptor expression and associated transcriptional activity under standard cell growth condition (i.e. exponential cell growth). The ligand-induced cell growth modulation was also assessed.

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Materials and methods

Chemicals

L-[^{35}S]-methionine (>1000 Ci/mmol), [^{3}H]-E_2 (88 Ci/mmol), [^{32}P]-dCTP were purchased from Amersham Biosciences (Buckinghamshire, UK). E_2, 4-hydroxytamoxifen (4-OH-TAM), PMSF (phenylmethylsulfonyl fluoride), TPCK (tosyl-L-phenylalanine-chloromethylketone) and agarose-bound anti-rabbit IgG antibody were from Sigma (St Louis, MO) whereas ICI 182, 780 was provided by Tocris (Illkirch, France). F-10 mouse monoclonal anti-human ERα antibody raised against F domain, D-12 mouse monoclonal anti-human ERα antibody raised against a recombinant protein corresponding to amino acids 2-185 mapping at the A/B domain of ERα, and HC-20 rabbit polyclonal anti-human ERα antibody raised against F domain were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). BCA protein assay kit, peroxidase-labeled goat anti-mouse IgG antibody was obtained from Pierce (Rockford, IL).

Cell culture

MCF-7 cells were routinely grown in basal EMEM medium supplemented with 10% inactivated FCS (fetal calf serum), 100 UI/ml streptomycin and 100 μg/ml penicillin. For experiments, cells were cultured in basal EMEM medium without phenol red and supplemented with 10% inactivated and charcoal-dextran stripped FCS.

Determination of the cytotoxic effects

MCF-7 cells were seeded in 96-well microtitration plates (1000 cells/well) to maintain cell growth during the whole duration of the experiment. Forty-eight hours later, cells were exposed to single agent (E_2; ICI 182,780; 4-OH-TAM); concentration ranges were as follows: l nM < [E_2] < 100 nM, 1 μM < [ICI 182,780] < 100 μM and 1 μM < [4-OH-TAM] < 100 μM.

Irradiation was performed, at room temperature, 48 h after plating using high energy photons from a linear accelerator 18 MV (Clinac, Varian Medical Systems) with 4 Gy/min. Medium was removed and replaced by fresh medium at time of irradiation. Dose effect curves were established using a total of 7 doses: 0.5, 1, 3, 5, 8, 10, 15 Gy. Cells were maintained in basal EMEM supplemented with 10% inactivated and charcoal-dextran stripped FCS during all radiation exposures.

At the end of the experiments, cells were gently washed once with PBS, fixed with 1% glutaraldehyde/PBS (15 min, 20 °C) and stained with 0.1% crystal violet (w/v in ddH_2O) (30 min, 20 °C). Excess of crystal violet dye was then removed by three washes of running tap water (15 min, 20 °C) and cells were lysed with 0.2% Triton X-100 (v/v in ddH_2O) (90 min, 20 °C, under agitation). The absorbance was measured at 550 nm using Microplate Autoreader EL309 (BIO-TEK Instruments).

Assessment of the effect of compounds combinations and/or radiation by isobolographic method for drug associations ICI 182,780 or 4-OH-TAM and/or E_2 in combination with γ-ray irradiation

Dose–response interactions between ICI 182,780 or 4-OH-TAM and/or E_2 in combination with ionizing radiations at 30, 50 and 75% cell growth inhibition ((IC30), (IC50) and (IC75)) were evaluated using crystal violet dye and results were analyzed by the classical isobolographic method described by Steel and Peckham [11]. The theoretical basis and procedure of the isobologram method have been described in details [12]. For a given level of efficacy (% survival) an 'envelope of additivity' curve was calculated from the dose effect curves of each compound (or drug combination) and from the dose effect curves of ionizing radiations (three doses). The coordinates of the experimental point are the drug concentration and the radiation dose which, when combined, give the level of efficacy. If the experimental point falls above, beyond or under the limits of the envelope of additivity, compounds and radiation combination give rise to antagonistic (Ant), additive (+) or synergistic (Syn) effects, respectively.

Cell cycle analysis

MCF-7 cells were maintained 48 h in estrogen-free medium before treatment. At the end of the experiment cells were trypsinized and washed twice with PBS. Cells were mixed thoroughly and stained using Coulter DNA-Prep reagent kit (Beckman Coulter, FL). Briefly, cells were resuspended in 50 μl of reagent A (15 s, 20 °C, under vortex agitation) and incubated in staining solution (950 μl of reagent B). After incubation (2 h, 4 °C, dark) cell cycle was analyzed with a Beckman FACS calibur analyzer and WinCycle software (Phoenix Flow Systems, San Diego, CA).

Determination of apoptosis

Apoptosis was determined by Annexin V staining (Biosource, Belgium). After treatment, cells were trypsinized and rinsed twice with PBS. Cell pellet was then incubate in Annexin V binding buffer in presence of 5 μl of Annexin V solution and 10 μl of Propidium iodide solution (15 min, 20 °C, in the dark). Staining solution was then discarded and replaced by Annexin V binding buffer. Annexin V positive, Propidium iodide negative cells (i.e. apoptotic cell) were detected by flow cytometry analysis using coulter xl cytometer.

ER binding determination

ER binding was measured by whole cell binding assays [13]. At the end of the treatment, cells were incubated

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with increasing concentrations of [^{3}H]-E_2 (10^{-9} M to 5×10^{-11} M) with or without a 500-fold excess of unlabeled E_2 (1 h at 37 °C). Cells were then washed twice with PBS buffer, bound [^{3}H]-E_2 was extracted in 250 µl ethanol (20 min, 20 °C) and radioactivity was measured by scintillation counting. Results were analyzed by Scatchard plot and the binding capacity (B_{max}) was expressed in fmol/mg of protein (measured in cell extracts by BCA protein assay kit, Pierce).

EIA measurement of ERα

Total cellular extracts were used for determination ERα content. MCF-7 cells were plated in 175 cm^2 Pétri dishes. At the end of the treatment, cells were washed twice in Hank's balance salt solution (HBSS), harvested by incubation with 1 mM EDTA, pelted by centrifugation (800 × g, 10 min, 4 °C) and washed twice in phosphate buffer (10 mM K_2HPO_4, pH 7.4, 10 mM thioglycerol, 1.5 mM EDTA, 10% glycerol). Cells were then homogenized in phosphate buffer with a Teflon glass potter and extracts were clarified by ultracentrifugation (100,000 × g, 30 min, 4 °C). ERα amounts were measured by the Abbot Enzyme Immunoassay (ER-EIA), according to the manufacturer's instructions.

Western blots

Western blots were performed as previously described [14]. Briefly, cell cultures were washed with TBS and lysed for 30 min at 4 °C in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF, 0.6 mM PMSF, 0.1 mM orthovanadate, 0.3 mM TPCK). Each sample (20 µg) was then loaded onto 5% stacking/12% running SDS polyacrylamide gel and transferred onto nitrocellulose membrane. Blots were incubated with primary antibody (1:1000 dilution, overnight, 4 °C). Detection was performed using a goat anti-mouse secondary antibody (1:2000 dilution, 1.5 h, room temperature) and an Western Pico Detection system.

Assessment of ER turnover

MCF-7 cells were plated in 100 mm Ø Pétri dishes (2×10^5 cells per dish). At the end of the experiment, cells were fed with MEM without L-methionine (2 h, 37 °C) and then exposed to 10 nM L-[^{35}S]-methionine (3 h, 37 °C). Five hundred micrograms of total protein extracts were submitted to immunoprecipitation [14]. Briefly, pre-cleared samples were incubated with a ER polyclonal antibody (HC-20, 2 µg, overnight, 4 °C) and the ER-antibody complexes were precipitated by incubation with an anti-rabbit agarose-bound IgG (45 µl, 2 h, 4 °C); Denatured samples were submitted to SDS–PAGE electrophoresis (4% stacking/12% running). Gels were then fixed (acetic acid 10%, methanol 40%, distilled water 50%) (30 min, 20 °C) and washed with distilled water (30 min, 20 °C). Radioactive signals were amplified by salicylic acid solution (1 M salicylic acid, 40% ethanol in distilled water) (90 min, 20 °C). Finally, gels were dried and submitted to fluorography to detect radio-labeled ER bands (67 kDa) (3 days, −80 °C) with hyperfilm MP (Amersham Biosciences, Buckinghamshire, UK).

After labeling (as described above), cells were rinsed twice and allowed to grow in fresh medium containing unlabeled methionine for 0, 1, 2 or 3 h before harvesting (chase experiments). Remaining [^{35}S]-labeled ERα were quantified as describe above.

Northern blot analysis

Northern blot analysis was performed as previously described [15]. At the end of the experiment, total RNA was extracted with TriPure according to the instructions of the manufacturer (Roche). Total RNA (15 µg) was separated on a 1% agarose gel in 2.22% formaldehyde, 0.02 M 3-(*N*-morpholino) propane sulfonic acid (MOPS) and 1 mM EDTA before transfer onto a nylon membrane (*Hybond-N*, Amersham) and UV-cross linked. Pre-hybridization (4 h) and hybridization (18 h) were performed at 42 °C (at room temperature when using the 28S ribosomal oligonucleotide) in 50% formamide, 5 × SSPE (20 × SSPE = 0.2 M phosphate buffer (pH 7.4), 2.98 M NaCl, 0.02 M EDTA), 0.1% SDS, 10% 5 × Denhard, 5% dextran sulfate, and 100 µg/ml sheared salmon sperm DNA. Hybridized membranes with TFF1 cDNA probe (probe was obtained from American Type Culture Collection) were washed twice at room temperature in 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.1% SDS, followed by three washes in 1 × SSC 0.1% SDS at 65 °C. The filters hybridized with the 28S ribosomal RNA oligonucleotide probe (Clontech) were washed only at room temperature in 2 × SSC 0.1% SDS. To account for variations in RNA loading, TFF1 values were normalized to 28S rRNA values. All membranes were exposed in autoradiography for various periods of time to ensure that only signals obtained in the linear range of film sensitivity were quantified. To rehybridize filters, former probes were first removed by incubating the membranes in pure water at 85 °C.

NASBA analysis

NASBA analyses were kindly performed by BioMérieux (Lyon, France). Briefly, 5 ng of RNA was added to 10 µl of NASBA buffer (final concentration in 20 µl reaction mixture: 40 mM Tris HCl, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 5 mM dithiothreitol, 15% v/v DMSO, 1 mM of each dNTP, 2 mM of each NTP, 0.2 µM of ESR1 primers, 0.2 µM of PPIB primers and 0.1 µM of each gene specific molecular beacon), pre-incubated at 65 °C for 2 min, followed by 2 min at 41 °C. Five µl of enzyme mix (0.08 U RNase H, 32 U T7-RNA polymerase, 6.4 U RT) was then added to start the RNA amplification, and incubated at 41 °C for

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90 min. ESR1 mRNA levels were normalized to PPIB mRNA levels. The GenBank accession numbers were X03635 for ESR1 and M60857 for PPIB.

Statistical analysis

Differences between the mean values were evaluated using either one-way ANOVA with Tukey's test or one-way ANOVA on ranks with Dunnetts' or Student–Newman–Keuls test, according to data distribution. $p = 0.05$ was considered as statistically significant. All analyses were carried out with the SPSS software (Paris, France).

Results

Impact of ionizing radiation on ERα content

Effect of radiation on [^{3}H]-E_2 binding parameters in MCF-7 cells (B_{max}, Kd) was analyzed by Scatchard plot (Figure 1a). A time-dependent decrease of binding capacity (B_{max}) was recorded in cells exposed to 8 Gy while B_{max} remained constant in non-irradiated cells. This inhibition became detectable 72 h after irradiation and reached 50% after 96 h. No statistically significant modification of B_{max} was observed for lower doses (3 and 5 Gy) (data not shown). Radiation did not affect the Kd value along the experiment, which remained around 1 nM indicating no change of the binding affinity of the remaining receptor for the hormone.

Influence of radiation on ERα content was then evaluated by EIA (Figure 1b) and Western blot (Figure 2) at the most efficient γ-rays dose of 8 Gy. EIA values indicated an ER loss (30% at 72 h and 50% at 96 h post-radiation, $p = 0.01$ and 0.00001, respectively) which was confirmed by Western blot analyses using F-10 antibody (raised against N-terminal domain of ERα). Indeed, densitometric quantification of the ERα (67 kDa) gave similar results as EIA quantification (significant decrease at 72 and 96 h with $p = 0.001$ and 0.001, respectively; Figure 2). Same results were obtained using antibody raised against C-terminal domain (D12 antibody) (data not shown), excluding the possibility of protein cleavage. Altogether our results indicated that 8 Gy radiation decreases ERα protein level in MCF-7 cells.

Ionizing radiation decreases ERα synthesis

In order to determine whether the 8 Gy radiation induced ERα loss was due to an inhibition of its synthesis or an accelerated degradation, receptor turn over rate was quantified by a [^{35}S]-methionine labeling

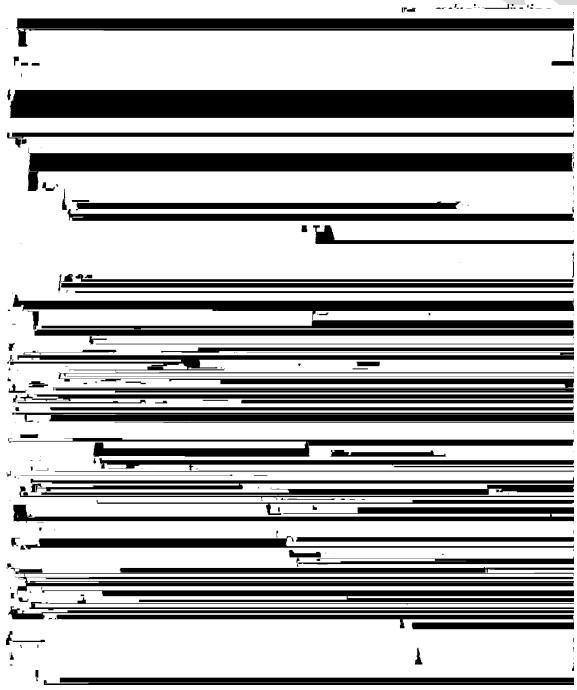

Figure 1. Effects of 8 Gy on the ER binding site content (B_{max}) and ERα expression. (a) Cells were irradiated 24, 48, 72, 96 h prior determination of ER binding site constant (B_{max}). B_{max} were obtained from Scatchard plot analysis and were expressed in % of control (= non-irradiated cells at time 0, $B_{max} = 526 \pm 37$ fmol/mg of proteins). Data refer to the mean value of three independent experiments performed in duplicate. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$. (b) Cells were cultured up to 96 h after irradiation and whole cell ERα content was measured by EIA. Results were expressed in % of control (= non-irradiated cells at time 0, control = 575 ± 25.4 fmol/mg of proteins). Data refer to the mean value of three independent experiments performed in duplicate. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

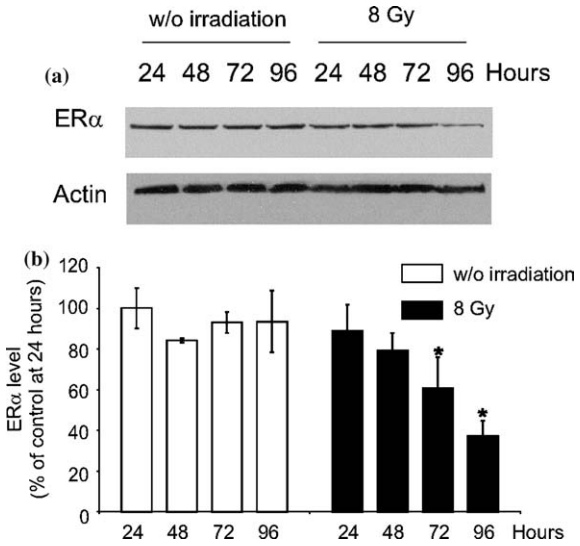

Figure 2. Western blot analysis of ionizing radiations on the ERα expression in MCF-7 cells. (a) ERα expression were detected 24–96 h post-irradiation by Western blot. Immunoblotting were performed using antibodies raised against N_T or C_T of ERα protein. Immunoblots were representative of three independent experiments. Equal loading was controlled by β-actin probe (see Materials and methods). (b) Immunoblots were submitted to densitometry analysis using Bio-Rad Quantityone Software. Results were representative of the three independent immunoblot analyses and expressed as percentage of control (non-irradiated cells at 24 h) corrected by actin value for each lane. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

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method (Figure 3). Radiation provoked a slight decrease of [^{35}S]-methionine ERα labeling (25%, 72 h post-irradiation, $p = 0.01$), which was more pronounced after 96 h (45%, $p = 0.001$). This ERα labeling decrease was not abolished by a proteasome inhibitor (10^{-6} M of MG-132, data not shown) suggesting that radiation affected ERα synthesis rather than its degradation by ubiquitin/proteasome pathway. In agreement with this view, pulse-chase experiment performed on [^{35}S]-methionine pre-labeled cells (Figure 4) failed to show any faster elimination of the remaining labeled ER after irradiation (maintenance of a 3 h period for the nearly total elimination of the receptor).

We next examined the effect of ionizing radiations on ERα mRNA expression by NASBA analysis (Figure 5). While no significant modification in ERα mRNA amount was observed at 72 h, 8 Gy induced a 50% decrease of ERα mRNA at 96 h as compared to non-irradiated cells (control), confirming that ionizing radiation affects ERα production.

Ionizing radiation fails to affect ERα transcriptional activity of the remaining receptor

Expression of TFF1 (pS2) (ERα reporter gene) was measured to evaluate the potential impact of ionizing radiation on its transcriptional activity. A slight increase of TFF1 mRNA levels was detected in basal conditions both at 72 and 96 h post-irradiation as already reported by Balcer-Kubiczek et al. [16]. A decrease of E_2-induced TFF1 level at 96 h closely related to the radiation induced ERα loss was also found, indicating that radiation did not modify transcriptional activity of the remaining receptors (Figure 6).

Ionizing radiation decreases E_2-induced cell growth

Potential impact of radiation on cell growth response to E2, 4-OH-TAM and ICI 182,780 was evaluated. As shown in Figure 7, 96 h post-irradiation, E_2-induced cell growth was significantly decreased by 33, 45 and 66% at 3, 5 and 8 Gy respectively. Moreover, growth rate of such E_2-exposed cells over passed the basal growth rate in irradiated cells, indicating that the hormone rescued cell growth. This growth rescuing action was abrogated by SERM or pure anti-estrogen (4-OH-TAM, ICI 182,780; at 100 nM) suggesting that it was relevant to a regulatory mechanism involving ER. The irradiation did not affect the growth inhibition by 4-OH-TAM or ICI 182,780 when tested alone (Figure 7). Interestingly, the protective effect of 17-β-estradiol seems to be

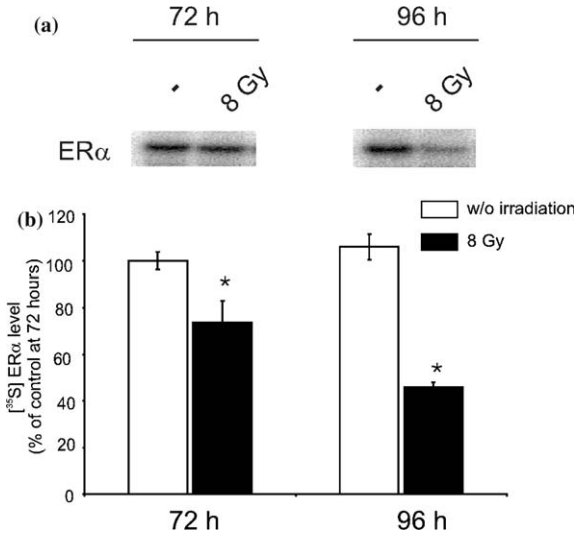

Figure 3. Effect of ionizing radiations on ERα protein synthesis in MCF-7 cells. 72 or 96 h prior irradiation, MCF-7 were incubated with 10 mM [^{35}S]-methionine for 3 h. At the end of the incubation, cells were lysed and immunoprecipitated ER was submitted to SDS–PAGE electrophoresis and fluorography. (a) Representative fluorography. (b) Densitometry analysis of three independent fluorography. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

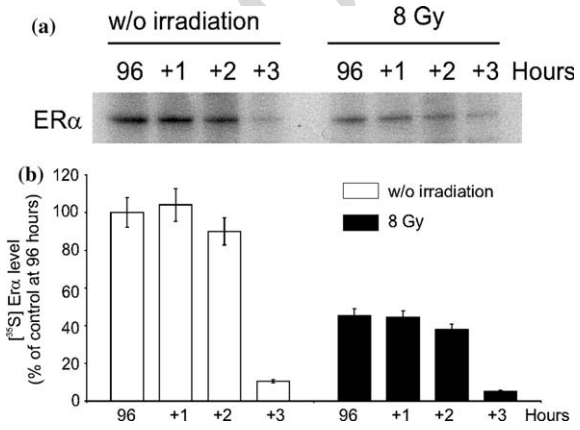

Figure 4. Effect of ionizing radiations on ERα protein degradation in MCF-7 cells. 72 or 96 h prior irradiation, MCF-7 were incubated with 10 mM [^{35}S]-methionine for 3 h. At the end of the incubation, cells were rinsed twice with non-radioactive medium and allowed to grow. Cells were then lysed and immunoprecipitated ER was submitted to SDS–PAGE electrophoresis and fluorography after 1, 2 or 3 h. (a) Representative fluorography. (b) Densitometry analysis of three independent fluorography. Statistical significance of data was calculated by ANOVA one way (irradiated and non-irradiated sample for each experimental condition), $*p < 0.05$.

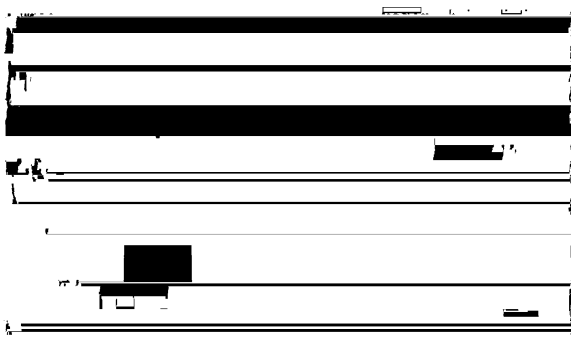

Figure 5. Effect of ionizing radiations on ERα mRNA amount. ERα mRNA levels were evaluated by NASBA after 72 or 96 h post-irradiation. Results were representative of the two independent analyses and expressed as percentage of control (non-irradiated cells) corrected by PPIB mRNA amount for each sample. Statistical significance of data was calculated by student *t*-test (irradiated versus non-irradiated sample for each experimental condition), $*p < 0.05$.

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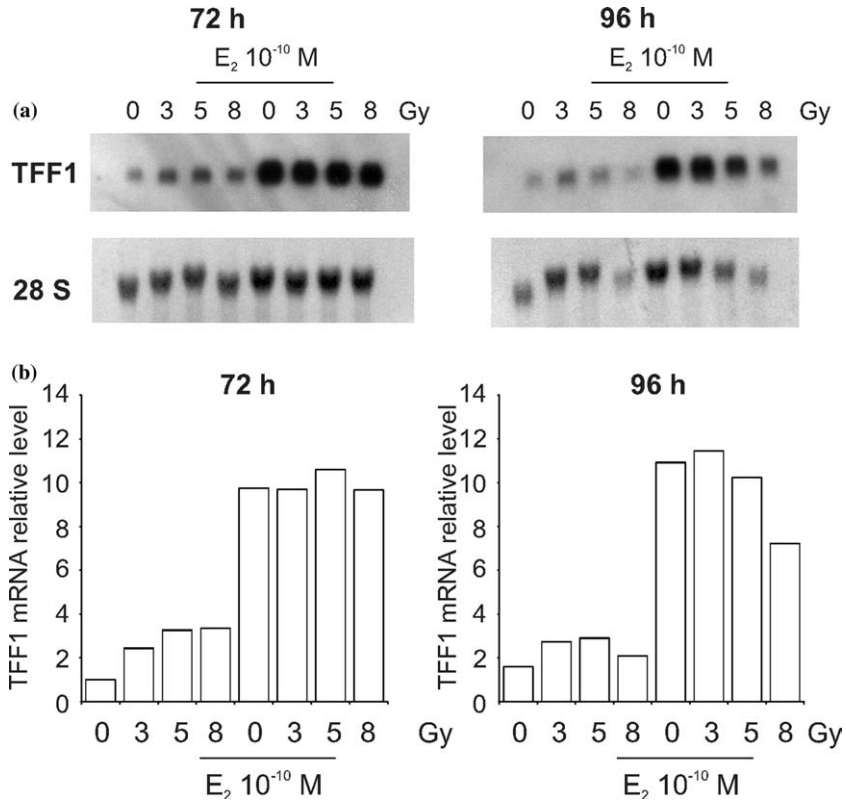

Figure 6. Effect of ionizing radiations on expression of TFF1. MCF-7 cells were cultured for 8 h without or with 10^{-10} M of E_2 prior to the end of experiments (72 or 96 h post-irradiation). (a) Basal rate and E_2-induced level were evaluated by Northern blot. The 28S levels are shown as internal control. Results representative of two independent experiments. (b) Results from densitometry analysis of the data shown in 'a' (normalized by the 28S RNA value).

maintained, even 10 days post-irradiation as measured in clonogenic assay. Indeed, at 8 Gy, MCF-7 cells were only able to grown in colonies in presence of 0.1 nM 17-β-estradiol; at lower irradiation doses, the hormone increased the number of colonies (data not shown).

The combination index (CI) at 30, 50 and 75% cell lethality are given in Table 1. Combination of ionizing radiations and E_2 conferred a strong antagonistic effect, while combinations with 4-OH-TAM and ICI 182,780 gave more often additive effects for 3 and 5 Gy and synergistic effects for 8 Gy.

17-β-estradiol impedes radiation cell cycle blockade

The effects of E_2 in cell cycle progression and apoptosis induction were determined after 8 Gy γ-rays exposure. As shown in Figure 8, radiation increased the percentage of cells in G0/G1 and G2/M after 48 h, indicating a cell cycle blockade in G0/G1 and G2/M phase as previously described [17]. 17-β-estradiol abrogated cell cycle blockade. Moreover, it still allowed cell cycle progression but to a lower extend than in non-irradiated cells. No significant modification of apoptosis induction under ionizing radiation was recorded both in the absence or presence of E_2 (Table 2); confirming the lack of potential correlation between radiosensitivity and apoptosis induction [17,18].

Discussion

The data reported here reveals two major findings: (1) 8 Gy-ionizing radiation induces a decrease of ERα synthesis but has no impact on receptor functions in MCF-7 breast cancer cells. Residual receptors seem to be unaffected, leading to the maintenance of a cell sensitivity to (anti)-estrogenic stimulation. (2) 17-β-estradiol may impede the cell growth inhibitory effect of 8 Gy-ionizing radiation.

Previous studies have revealed a loss of estrogen binding capacity without evidence of ER loss [9]. Difference in experimental protocols (i.e. radiation after plating (previous) versus during exponential growth phase (present)) may explain this partial discrepancy. Potential reasons for ER loss are multiple. Ionizing radiations are essentially characterized by DNA lesions which activate ATM/BRCA1 reparation pathways. Indeed, ATM and BRCA1 act as sensors of genetic alterations and activate downstream targets, like P53 and $P21^{waf1/cip1}$, responsible of the cell cycle arrest in G0/G1 or G2/M phases [17]. Changes in these transduction pathways interfere with ERα expression and/or activation providing possible explanation of our data. Thus, BRCA1 is a major co-repressor of ERα transcriptional activity. It may act by decreasing ERα binding to its target promoters and/or also inhibit transcriptional activity of ERE (estrogen response element) bound

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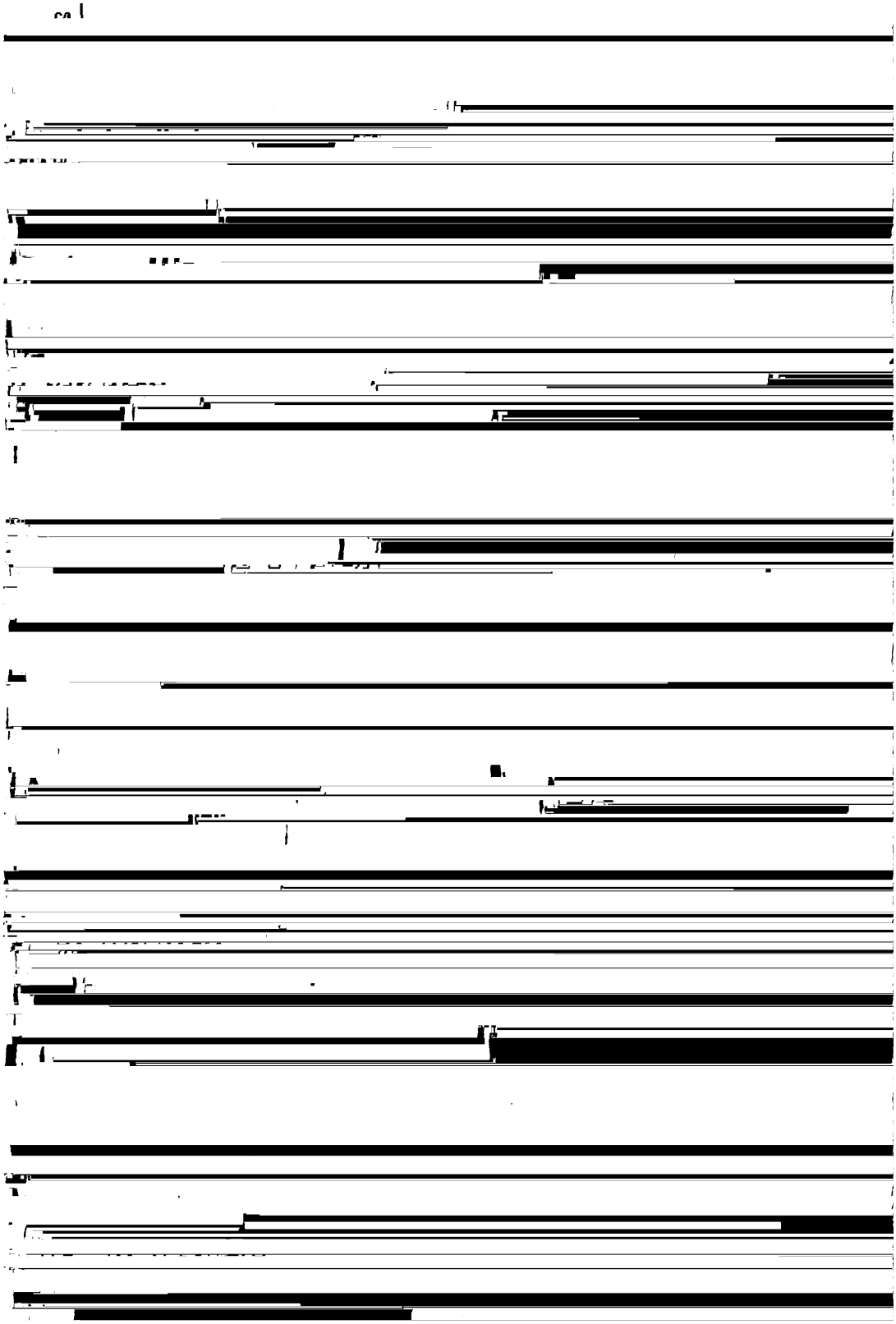

Figure 7. Effect of ionizing radiations on MCF-7 cell growth induced by E_2. Cells were irradiated (3, 5, 8 Gy) and subsequently maintained in culture for 96 h either in the absence or presence of estrogenic or/and anti-estrogenic ligands (E_2 at 0.1 nM, 4-OH-Tam at 100 nM, ICI 182,780 at 100 nM). Non-irradiated cells were cultured in parallel. Growth was estimated by crystal violet staining each day after irradiation. Results were expressed as a relative cell growth. Cell number at the time of irradiation was arbitrary defined as 1. Results are representative of three independent experiments performed at least six times.

receptor [19]. The decrease of ERα expression and activity are late events occurring after the cell cycle arrest exclude BRCA1-related effects. On the other hand, Angeloni et al. [20] recently demonstrated that p53

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Table 1. Results of isobolographic analyses of ionizing radiation estrogenic or anti-estrogenic drug interactions on MCF-7 cell line

Combined chemotherapy	Rγ Gy	30% surv	50% surv	75% surv	Mean
ICI 182780	3	Syn	Syn	+	Syn
	5	Syn	+	+	Syn
	8	+	Syn	Syn	Syn
4-OH-TAM	3	+	+	+	+
	5	Syn	Syn	+	Syn
	8	Syn	Syn	Syn	Syn
E_2	3	Ant	Ant	Ant	Ant
	5	Ant	Ant	Ant	Ant
	8	Ant	Ant	Ant	Ant
E_2 + ICI 182780	3	+	+	Syn	+
	5	Syn	+	Syn	Syn
	8	Syn	Syn	Syn	Syn
E_2 + 4-OH-TAM	3	+	+	Syn	+
	5	+	+	Syn	+
	8	Syn	Syn	Syn	Syn

Isobolographic interpretation at 30, 50 and 75% growth inhibition (results from three separate experiments). Mean were calculated from isobolographic values achieved at IC30, IC40, IC50, IC60 and IC75. Syn = synergistic effect; ant = antagonistic effect; + = additive effects; surv = survival.

activation leads to a decrease of ERα expression in breast cancer cells, suggesting a potential implication of this protein.

ERα loss may also be a consequence of the cell cycle arrest. ERα expression in breast cancer cells MCF-7 is indeed dependent of cell cycle progression. Jakesz et al. [13] first revealed that G1 cell arrest decreases ERα content. Recently, de Graffenried et al. [21] demonstrated that ERα expression is dependent of SP1, a transcription factor tightly regulated during cell cycle progression. On the other hand, cell cycle key proteins are under control of ubiquitin-like proteins which modulate their proteosomal degradation. Interestingly, level and activity of ERα and associated regulatory proteins are dependent of ubiquitin-like degradation pathways [22]. Thus, neddylation (a degradation pathway involved in cell cycle progression) also targets ERα and SRC-1 (ERα co-activator) to inhibit their activities. However, as shown in the present study, radiation-induced ERα decrease is due to a progressive arrest of its synthesis rather than to an enhancement of proteasomal degradation (MG-132 did not abrogate its loss) which seems to reject the implication of proteasome in radiation-induced ER loss.

It should be stressed that ionizing radiation inhibits E_2-induced cell growth independently of its ability to decrease ERα content since growth inhibition occurs largely before ER loss. Nevertheless, E_2 induced cell growth remains specific to ERα activation since they are totally blocked by SERM and anti-estrogens. Actually, ionizing radiation may interfere with E_2-induced cell growth by influencing E_2-dependent pathways such membrane signaling pathways or P53/P21$^{Waf\text{-}1/Cip1}$ axis [23,224]. Our results do not allow to conclude upon the potential implication of these cross-talks and further investigations are needed.

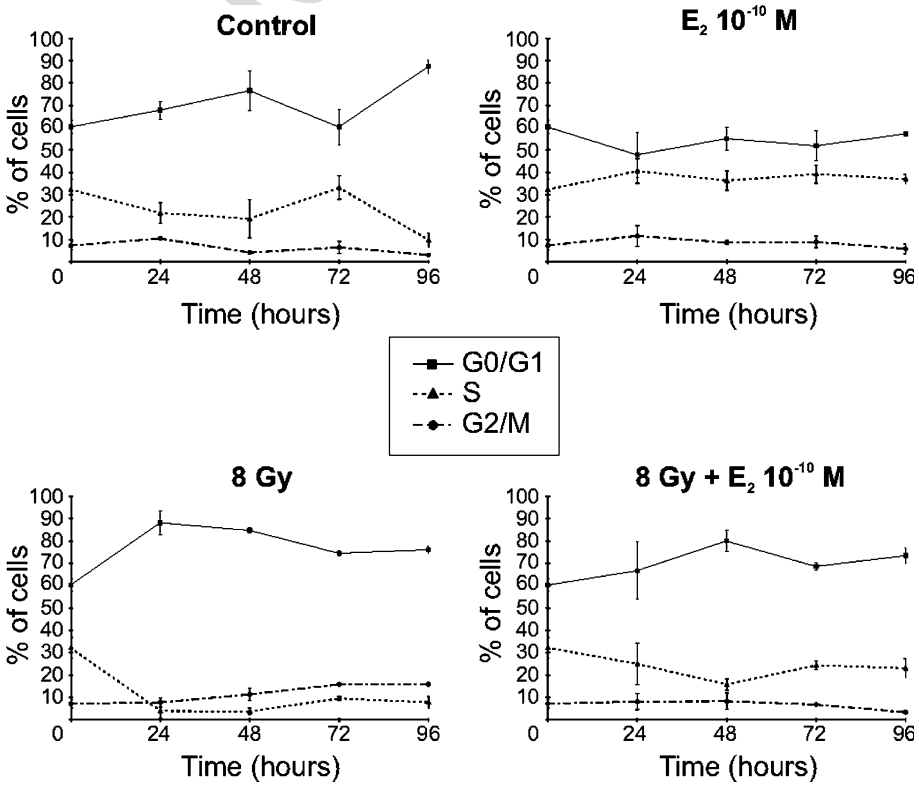

Figure 8. Effect of E_2 on cell cycle in MCF-7 cells. Cells were irradiated at 8 Gy and subsequently maintained in culture up to 96 h. Cell cycle was determined each day after radiation exposure by flow cytometry analysis. Results are representative of three independent experiments.

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Table 2. Apoptosis determination in MCF-7 breast cancer cells

		0 h	24 h	48 h	72 h	96 h
Non-irradiated	–	2.12 ±1.15	2.51 ± 1.54	1.18 ± 0.76	0.70 ± 0.17	1.67 ± 0.14
	E_2	–	3.55 ± 2.79	0.81 ± 0.69	0.21 ± 0.03	2.42 ± 0.19
8 Gy	–	–	2.56 ± 0.65	0.68 ± 0.50	0.57 ± 0.19	2.16 ± 0.73
	E_2	–	4.23 ± 1.11	0.78 ± 0.72	0.33 ± 0.12	3.34 ± 2.56

Percent of annexin V positive, propidium iodide negative cells (i.e. % of apoptotic cells) were detected by flow cytometry analysis. E_2: 17β-estradiol (1×10^{-10} M). Results are mean value of three independent experiments.

As compared to other experimental studies (reviewed in Schmilberger et al. [9]), our observations may be of clinical relevance. Association of SERM or anti-estrogen therapy before and concurrently to radiation may obviously block the effect of natural estrogen production; as a consequence, it may partially reduce the impact of a potential estrogen-associated radio-resistance. Clinical studies describing the sequence of endocrine therapy as tamoxifen or aromatase inhibitors and radiotherapy (concurrent versus sequential) are still rare, stressing the importance of our investigations. In retrospective studies with breast conservation in early stage breast cancer, Pierce et al. [25] and Christensen et al. [26] described that efficacy was not affected by the sequence of administration of tamoxifen and radiotherapy. In agreement with our experimental observations, the NASBP-B14 trial shows a significant decrease of breast relapse at 5 years with concomitant TAM treatment. On the other hand, results about toxicities are still closed up in debate but recent studies clearly demonstrated that toxic side effects are patient specific [8]. Furthermore concomitant treatment with pentoxifylline and alpha tocopherol treatment may avoid this toxic effects [27], raising the importance to pursue such investigations.

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