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Insulin and estrogen receptor ligand influence the FGF-2 activities in MCF-7 breast cancer cells

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Abstract

From the MCF-7 cell line we have developed, a human mammary cancer cell subline with the same karyotype as the mother strain and named MCF-7^{SF}, able to grow in serum-free chemically defined medium. This cell subline was firstly used to analyze the effect of basic fibroblast growth factor (FGF-2) in estrogen-receptor-positive human breast cancer cells. FGF-2 like estradiol is able to increase cell proliferation and pS2 expression but was also found to inhibit progesterone receptor (PR) expression. The anti-estrogen tamoxifen partly counteracts the effects of FGF-2 and to discriminate between its two main mediators (estrogen receptor vs. anti-estrogen binding site, AEBS) we compare the efficacies of pure anti-estrogen (ICI 182,780) and AEBS ligand (PBPE). It appears that pure anti-estrogen counteracts cell growth and pS2 effects of FGF-2 since AEBS ligand inhibits the cell growth but has no activity on pS2 expression. Secondly, adding insulin (10^{-6} M) in the culture medium induces a strong increase in cell proliferation, which then elicits an inhibitory effect of FGF-2 and addition of anti-estrogens, are less efficient to further decrease growth, since the effects of FGF-2 and anti-estrogens on pS2 expression are conserved.

Keywords: Breast cancer; FGF-2; Insulin; Estrogen; Anti-estrogen; AEBS

1. Introduction

In the quest to understand the regulation of breast cancer cell proliferation, the profusely documented studies on the estrogen-dependent MCF-7 cell line have focused on the forward approach of autocrine and paracrine mechanisms. It has been shown that MCF-7 cell proliferative activity is positively and negatively regulated by a combination of cytokines, the expression and secretion of which could be mediated by estrogen [1–4]. In this context, the growth proliferation activity of the basic fibroblast growth factor (FGF-2) has been the subject of various and conflicting observations [5–9]. Studies in this domain have mostly been performed using serum-dependent MCF-7 cells transferred from serum-containing to serum-free medium prior to the experiments. These conditions lead to a large variability of cellular responses depending on the type of serum, its concentration in the medium and the duration of starvation [10–12]. We therefore developed an MCF-7 cell subline able to grow in a chemically defined medium in conditions involving estradiol stimulation of cell growth. In this work the selected MCF-7 cell subline (named MCF-7^{SF}) presenting the same karyotype as its mother strain was used to investigate the biological effects of FGF-2 in the presence or absence of anti-estrogens. A comparative study was done on FGF-2 activities between the modulatory effects of pure anti-estrogens able to only bind the estrogen receptor [13] and selective ligands of the AEBS [14–17]. In parallel to the cell proliferation studies, we measured the expression of two protein markers of estrogen-dependence: the PR and pS2 [18].

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Abbreviations: AEBS, anti-estrogen binding site; PBPE, pyrolidino-benzyl-phenoxy-ethanamine; ER, estrogen receptor; PR, progesterone receptor; E2, 17β-estradiol; TAM, tamoxifen.

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2. Materials and methods

2.1. Hormone and growth factor

Diphenylmethane derivatives (PBPE) (AEBS ligands were synthesized in our laboratory) [19] E2 (Sigma), pure antagonist ICI 182,780 (ICI) and TAM (Sigma) were kept at −20° in ethanol solution at a concentration 1000 times that used in the experiments. The home made human recombinant form of FGF-2 (18 kDa), produced in *E. coli* [20] was stored at −20° in the presence of 0.5% bovine serum albumin in DME culture medium (Gibco) at 200 times the concentration used in the experiments. Radioactive [^{125}I]FGF-2 (specific activity: 1000 Ci/mmol) and [3,4,6,7-^{3}H]-estradiol (specific activity: 110 Ci/mmol) were from Amersham. Human insulin was from Novonordisk.

2.2. Maintenance of cell cultures

The MCF-7 cell lines were produced by the Michigan Cancer Foundation (Detroit, MI) and sent to us in their 275th passage. They had originally been grown in medium supplemented with 5% calf fetal serum and were gradually adapted to an RPMI 1640 medium containing phenol red, sodium bicarbonate (2 g/L), glutamine (2 mM), transferrin (10 mg/mL) and insulin (10^{-6} M) in the absence of serum and at pH 7.4, in a humid atmosphere containing 5% CO_2 and 95% air at 37°. The medium was changed every 2 days and the cells plated at subconfluency every 6 days.

2.3. Cell proliferation studies

Cells were used between passages 298 and 307. Cells in exponential growth phase (10^5 cells) were seeded in each 35-mm diameter Petri (Nunc) dish. Selective medium free of phenol red was added the day after plating and renewed every 2 days. The cells were removed by gentle trypsination then counted with a Coulter counter (Coultronics) hemocytometer. Three to five random counts were made the day after plating, to ensure reproducibility, and the experiment was only taken to completion when the plating efficiency was at least 90%.

2.4. pS2 and PR assays

The ELISA technique was used to assay both proteins. pS2 protein and PR were assayed according to the manufacturer's recommendations: CISbio industry and Abbot, respectively.

2.5. Estrogen receptor assays

E2 binding to ER was measured using a 105,000 *g* supernatant of cell extracts as previously described [21]. An ELISA assay was also carried out (in 0.4 M KCl containing buffer) as recommended by the manufacturer (Abbot).

2.6. FGF-2 binding experiments

Radio-labeling of FGF-2 with ^{125}I (specific activity 1000 Ci/mmol) and binding experiments at equilibrium using intact cells at 4° were carried out as described by Moscatelli [22].

2.7. Northern blot analysis of mRNA

The total RNAs (20 μg) were subjected to agarose (1%) gel electrophoresis in the presence of formaldehyde, transferred to a nylon membrane (Amersham), hybridized to pS2 using the corresponding probe then calibrated by means of the constant probe 36B4. The probes were labeled with [^{32}P]dCTP using a multi-primer labeling system (Amersham). Hybridization was continued for 18 hr at 42° in buffer containing 50% formamide, 5× SSPE, 2× Denhardt, 0.1% SDS containing 100 μg/mL of denatured herring sperm DNA (migration buffer 2×10^{-2} M MOPS, 10^{-3} M sodium acetate, 10^{-3} M EDTA, pH 8). The [^{32}P] radiolabeled bands were quantitated with a Molecular Dynamics Phosphorimager.

3. Results

3.1. Cell proliferation studies

The MCF-7^{SF} subline was selected by gradually lowering the serum concentration ratio in the culture medium. However, to ensure sufficient cell growth, transferrin and insulin were added (Section 2). Although the presence of transferrin appeared to be essential for cell life, insulin removal only led to considerably increased duplication time (see Table 1). To obtain sufficient cells quickly the MCF-7^{SF} subline was maintained in RPMI 1640 medium containing transferrin and insulin. After six passages in

Table 1
Doubling times of MCF-7^{SF}

RPMI 1640 + Tf	Control (hr)	+E2 10^{-8} M (hr)	+FGF-2 1 ng/mL (hr)	+E2 10^{-8} M + FGF-2 1 ng/mL (hr)
Without insulin	84 ± 7	51 ± 2	52 ± 2	36 ± 4
With insulin	38 ± 4	36 ± 4	52 ± 2	54 ± 5

Cells were plated at 10^5 per Petri dish. They had been grown for 5 days and the doubling time was determined using the formula: $t_2 = t \log^2/\log(nt/ni)$ where t_2 is the doubling time, nt is the cell number after 4 or 5 days treatments (with: control RPMI + transferrin, E2, FGF-2, E2 + FGF-2, in the absence or presence of insulin 10^{-6} M) and ni the cell number at the start of treatment. Each value is the mean ± SD of at least eight experiments, FGF-2 at 1 ng/mL = 5.6×10^{-11} M.

these conditions we observed a decrease or a loss of the estrogenic sensitivity of the cells, this disadvantage was counteracted by adding the low estrogenic activity carried by the phenol red contained in commercial RPMI 1640 (the same effect is obtained with estradiol at 0.5×10^{-10} M).

Thus to ensure reproducibility of the experiences, cells were routinely growth in RPMI 1640 medium containing phenol red, transferrin and insulin, the day after plating insulin and phenol red were withdrawn and each treatment was initiated on the third day.

As shown in Fig. 1 in the absence of insulin FGF-2 is an activator of proliferation and its activity is increased in the presence of E2. The saturation of the FGF effect was achieved at 1 ng/mL (5.6×10^{-11} M) and the dose effect giving 50% of the maximum activity was 0.2 ng/mL in the presence or absence of E2. The latter value gives a K_d of around 1.1×10^{-11} M for FGF-2 on its receptor. Pure anti-estrogens (ICI 182,780), tamoxifen and AEBS ligands are able to counteract this activity (Fig. 2).

The presence of insulin reverses the activity of FGF-2 which becomes an inhibitor of the cell growth (Fig. 3 and Table 1) whereas estradiol did not exhibit any significant activity. At the same time anti-estrogens become less powerful inhibitors of cell growth (Fig. 2). Interestingly, the FGF-2 concentration which inhibits 50% of the maximum effect is around 0.2 ng/mL corresponding to the same K_d (1.1×10^{-11} M) found for receptor(s) involved in the proliferation increase.

The cell duplication times calculated from growth experiments and reported in Table 1 evidence that insulin is the best enhancer of cell growth, FGF-2 and E2 alone are not as strong as insulin but when associated they can give the same proliferation rate as insulin. FGF-2 at its maximal effect produced the same doubling time in the presence (inhibitor) or absence (activator) of insulin.

A modulation of the FGF-2 and/or E2 receptors by the various treatments could explain the different regulations of cell growth observed. We therefore tested this possibility by assaying the corresponding receptors.

3.2. FGF-2 receptors

The FGF surface membrane receptors (FGF-R) are encoded by four homologous genes [23] and we investigate FGF-2 binding with this cellular model. The equilibrium dissociation constants of FGF-2, calculated with the Ligand program [24] (Fig. 4) were $K_{d1} = 8.6 \times 10^{-12}$ M for the high affinity-binding and $K_{d2} = 9.4 \times 10^{-10}$ M for the low-affinity binding accounted for the amino-glycans produced by the cell [25]. The numbers of radioactive FGF-2 binding sites were 5000 ± 3000 and $20{,}000 \pm 10{,}000$ sites/cell, respectively. Although the Scatchard analysis (Fig. 4) only shows two slopes we analyzed the repertoire of FGF-R by RT-PCR as previously described [26].

Five isoforms of FGFRs (FGFR1 IIIc; FGFR2 IIIb; FGFR2 IIIc; FGFR3 IIIc and FGFR4) were detected in

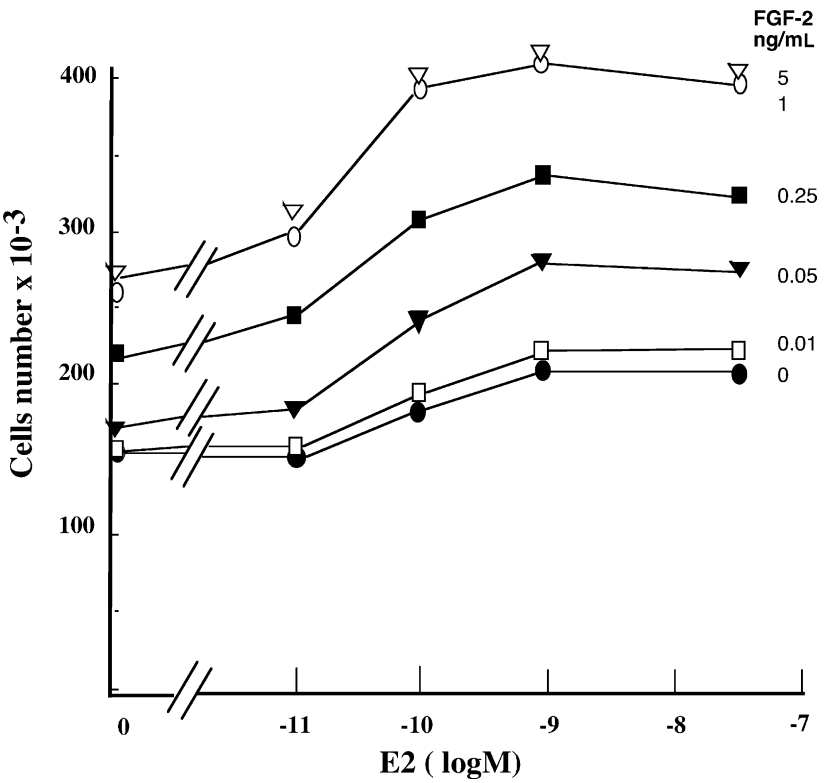

Fig. 1. Activities of the FGF-2 × E2 combination on cell proliferation 10^5 cells were seeded as described in Section 2 and counted after 4 days proliferation in the absence of insulin under FGF-2 treatments ((●), control; (□), 0.01 ng/mL; (▼), 0.05 ng/mL; (■), 0.25 ng/mL; (○), 0.1 ng/mL; (▽), 0.5 ng/mL) and in the presence of increasing E2 concentrations (from 10^{-11} to 5×10^{-8} M).

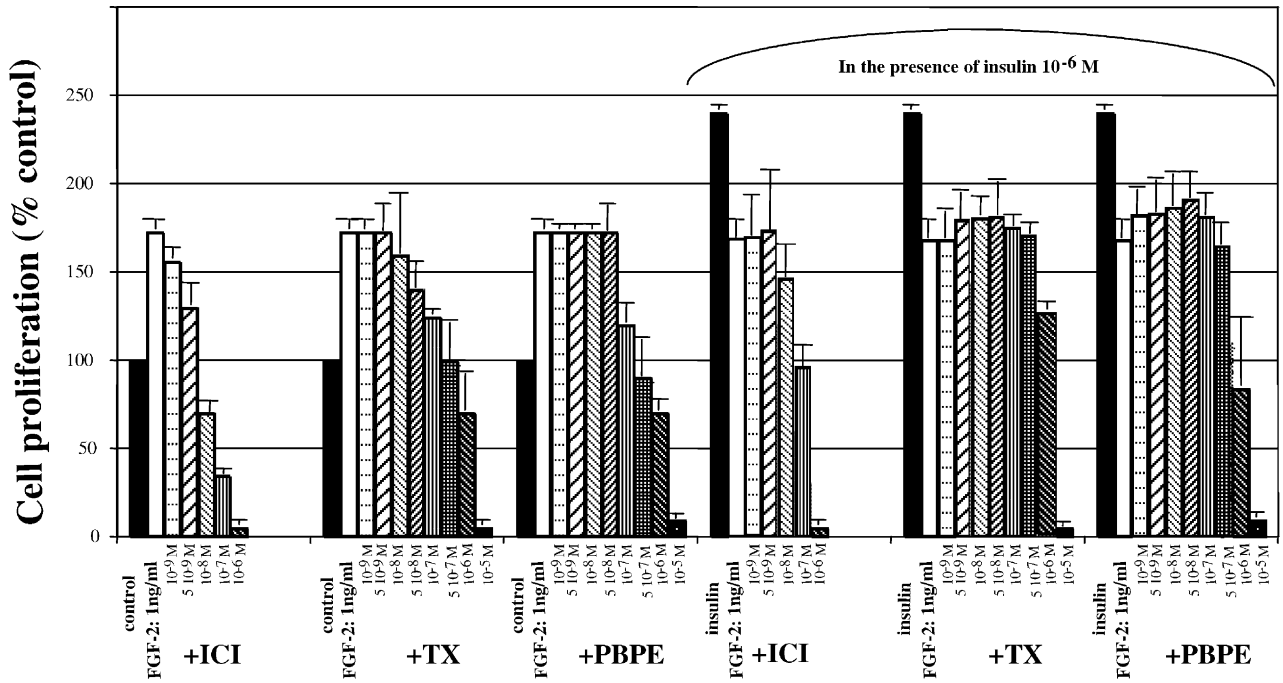

Fig. 2. Antagonistic activities of anti-estrogens on the FGF-2 cell growth control. Effect of anti-estrogens on the growth of MCF-7^{SF} under FGF-2 treatment. Results are expressed as the percent of the cell control (in black) obtained after 4 days proliferation in the minimum culture medium (RPMI 1640 in the absence of phenol red and insulin). On the left, cells were treated by one dose (1 ng/mL) of FGF-2 (in white) in the presence of increasing concentrations of anti-estrogens (from 10^{-9} to 10^{-5} M). On the right, the same experiment was carried out in the presence of 10^{-6} M insulin. Experiment were realized five to seven times in triplicate.

this MCF-7 subline however neither with RT-PCR nor with binding studies we were able to determine their relative concentrations. The same patterns of RT-PCR were observed whether insulin was present or not and whatever the treatment used. Concomitantly, Scatchard studies did not reveal variations either in the affinity or in the binding site number of the radioactive FGF-2 (data not shown).

3.3. Estrogen receptor α

Irrespective of the presence of insulin, the concentration of E2 binding sites in the soluble cell fraction was, unexpectedly, in an inverse relation with the concentration of FGF-2 added to the culture medium (Table 2). The dissociation constant at equilibrium was not significantly modified by the treatment. To estimate the crosstalk effect of estradiol and FGF-2 on the estrogen receptor concentration we used an immuno-enzymatic assay of ERα which showed that the reduced number of binding sites corre-

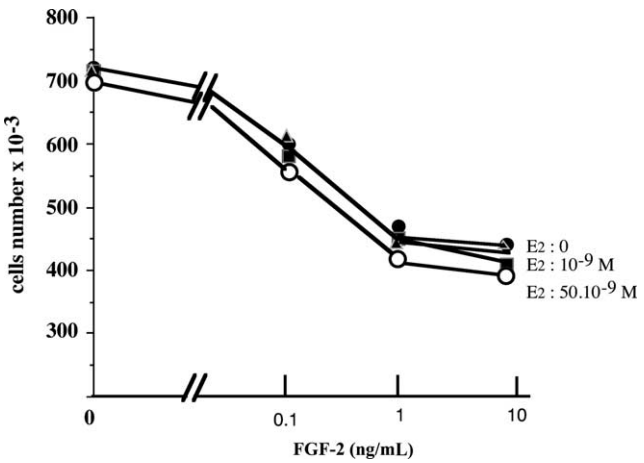

Fig. 3. Activities of the FGF-2 × E2 combination on cell proliferation in the presence of 10^{-6} M insulin. 10^5 cells were seeded as described in Section 2 and counted after 4 days proliferation in the presence of insulin under FGF-2 treatment and in the presence of various E2 concentrations.

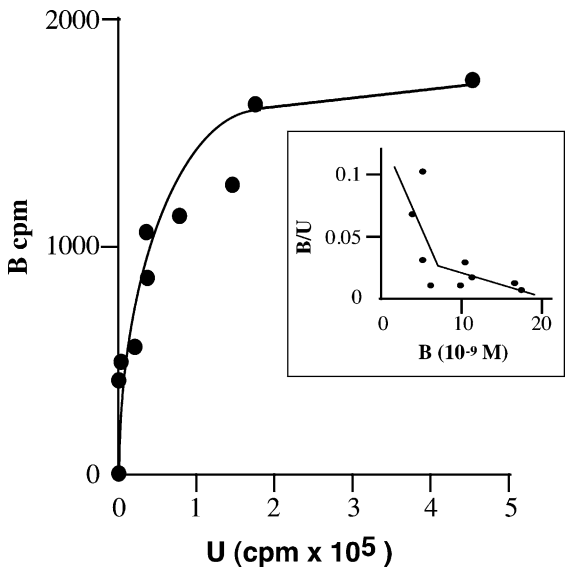

Fig. 4. Saturation binding of [^{125}I]FGF-2 at 4° in intact cells. Intact cells were submitted to increasing concentrations of [^{125}I]FGF-2 binding was carried out at 4°. The method used has been described by Moscatelli [22] and the binding parameters were obtained with the EBDA-Ligand program. Bound ligand (B); unbound (U).

Table 2
Estradiol binding properties

Medium = RPMI 1640 + transferin	$K_d \times 10^{-9}$ M	Sites/cell
With insulin	0.42 ± 0.2	10,100 ± 1,500
+FGF-2 = 0.05 ng/mL	0.26 ± 0.1	4900 ± 0500
+FGF-2 = 1 ng/mL	0.30 ± 0.18	2200 ± 0900
Without insulin	0.4 ± 0.25	7200 ± 1700
+FGF-2 = 0.05 ng/mL	0.23	3700
+FGF-2 = 1 ng/mL	0.30	1700

After 4 days of treatment with FGF-2 in the presence or absence of insulin, the cells were scraped off and binding studies carried out with increasing concentrations of [^{3}H]-estradiol (from 0.1 to 10 nM) and analyzed with EBDA-Ligand software. Each value is representative of four independent experiments done in duplicate. Experiments without insulin in the presence of FGF-2 were carried out only twice.

sponded to a reduction in the cellular concentration of the estrogen receptor protein (Fig. 5). Whatever the method used to measure estrogen receptor it appeared that insulin increased and FGF-2 decreased ER. Differences in the number and the variation of ER sites could be explained by the use of two buffers for the ER extractions, one containing KCl 0.4 M (Fig. 5, for the immuno-enzymatic assay) which is able to extract DNA bound ER from the nucleus and the other without KCl dissolving only DNA unbound receptor (Table 2, for the binding studies). Moreover, if ELISA is able to quantificate the ER protein, binding studies determined protein(s) which are able to bind [^{3}H]-estradiol. Northern blot analyses confirm a decrease in the ERα-mRNA under FGF-2 treatment (data not shown).

3.4. Modulation of progesterone receptor expression

PR is a clinical marker of the estrogen sensitivity of mammary tumors. Fig. 5 shows that PR production, in the presence or absence of insulin, was stimulated by E2 but inhibited by FGF-2, their effects on PR expression appeared antagonistic. Although this result is in good agreement with the cell growth inhibitory effect of FGF-2 in the presence of insulin, it appeared paradoxical with its stimulatory effect in the absence of insulin. However, FGF-2 effects are lower on PR than on ER expressions. Insulin alone increases the PR expression and a low additive effect is observed in the presence of estradiol. Measurements of the mRNA level by Northern blot analysis exhibit the same variations (data not shown). Tamoxifen and pure antagonist ICI 182,780 decrease the E2 effect proportionately to their respective affinity for ER and do not interfere with FGF activity. The AEBS ligand has no significant activity on PR regulation (data not shown).

3.5. Modulation of pS2 expression

The human pS2 gene, cloned from a bank consisting of E2-stimulated MCF-7 cells [27,28] codes a 7 kDa trefoil protein which is secreted and which has been suggested to have an anti-oncogenic role. Apart from the estrogen responsive elements, the promotor region of the gene has been shown to contain the activating nucleotide sequences regulated by EGF and FGF-2, phorbol esters, cHa-ras and c-jun [29]. In the MCF-7^{SF} model, E2 and FGF-2 increased the concentration of pS2 proteins in the

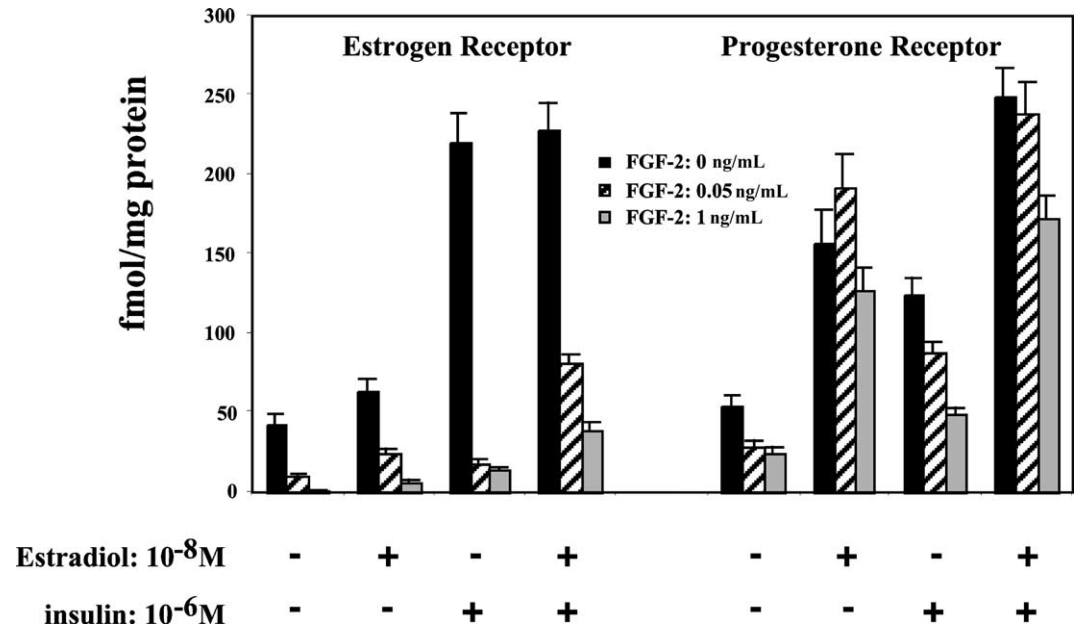

Fig. 5. Immuno-enzymatic measurements of ER and PR. The cells were seeded at 10^5 per dish deprived of phenol red and insulin for 2 days then treated with increasing concentration of FGF-2 in the presence or absence of estradiol or insulin for 4 days then ER and PR analyses were performed with ELISA kit as recommended by the manufacturer (Abbot). Results are expressed as fmol/mg protein, in our conditions with MCF-7^{SF} 50 fmol/mg protein correspond to 7000 molecules/cell.

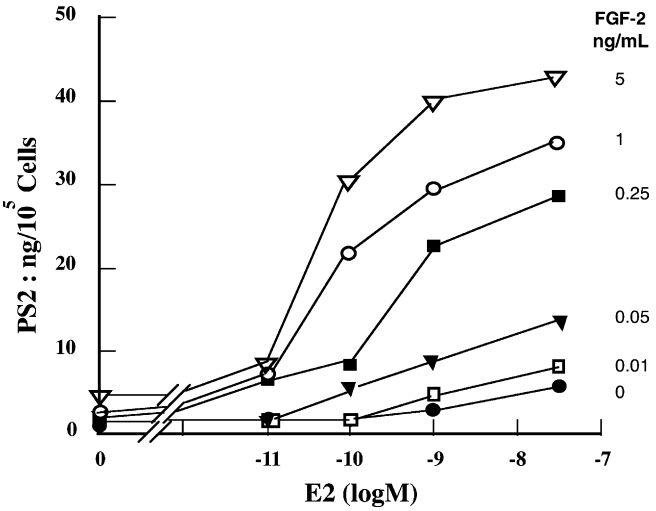

Fig. 6. Effects of E2 combined with FGF-2 on pS2 production. The cells were seeded at 10^5 per dish deprived of phenol red and insulin for 2 days then treated with E2 (from 10^{-11} to 5×10^{-8} M) and FGF-2 at increasing concentrations for 4 days ((●), control; (□), 0.01 ng/mL; (▼), 0.05 ng/mL; (■), 0.25 ng/mL; (○), 1 ng/mL; (▽), 5 ng/mL). The pS2 assay was carried out as described in Section 2, the cells were counted and the amounts of secreted pS2 (over the last 48 hr) per 10^5 cells determined.

extracellular medium both in the presence (data not shown) and absence of insulin (Fig. 6). These results reflect a modulation of the concentration of mRNA coding for pS2. Northern blot analysis using 36B4 as constant probe, showed variations in the relative concentrations of the two mRNA (36B4 and pS2) in relation to the different treatments (Fig. 7). Pure antagonist ICI 182,780 and tamoxifen reverse FGF-2 enhancement whereas AEBS ligand has no activity on the pS2 expression (Fig. 8). These observations are in agreement with a ligand independent

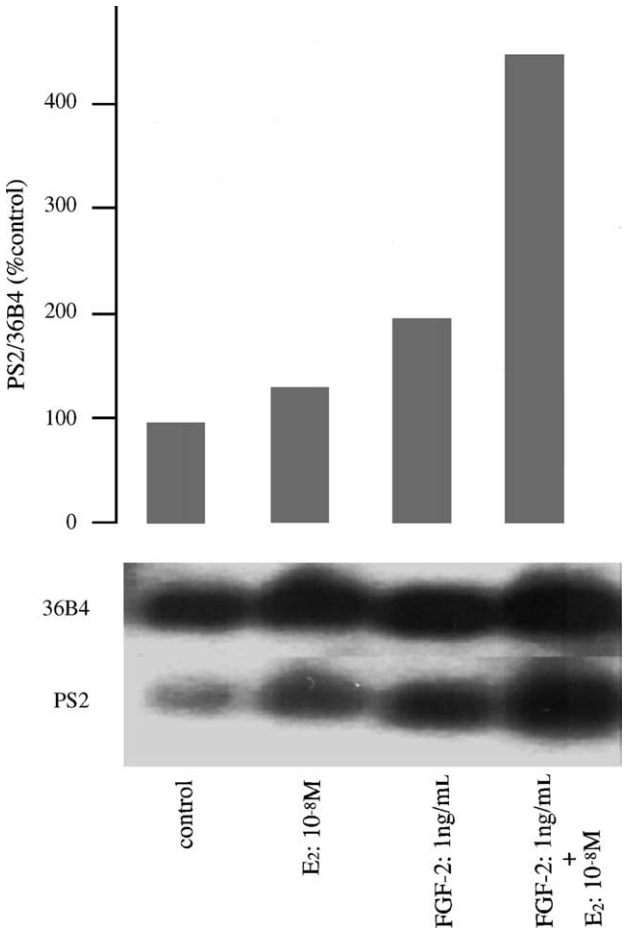

Fig. 7. Northern blot analysis of pS2 mRNA. Twenty micrograms of total RNA from treated cells in the absence of insulin (18 hr treatments) were loaded on agarose gel and Northern blot analysis carried out as described in Section 2. The figure is representative of one of four independent experiments.

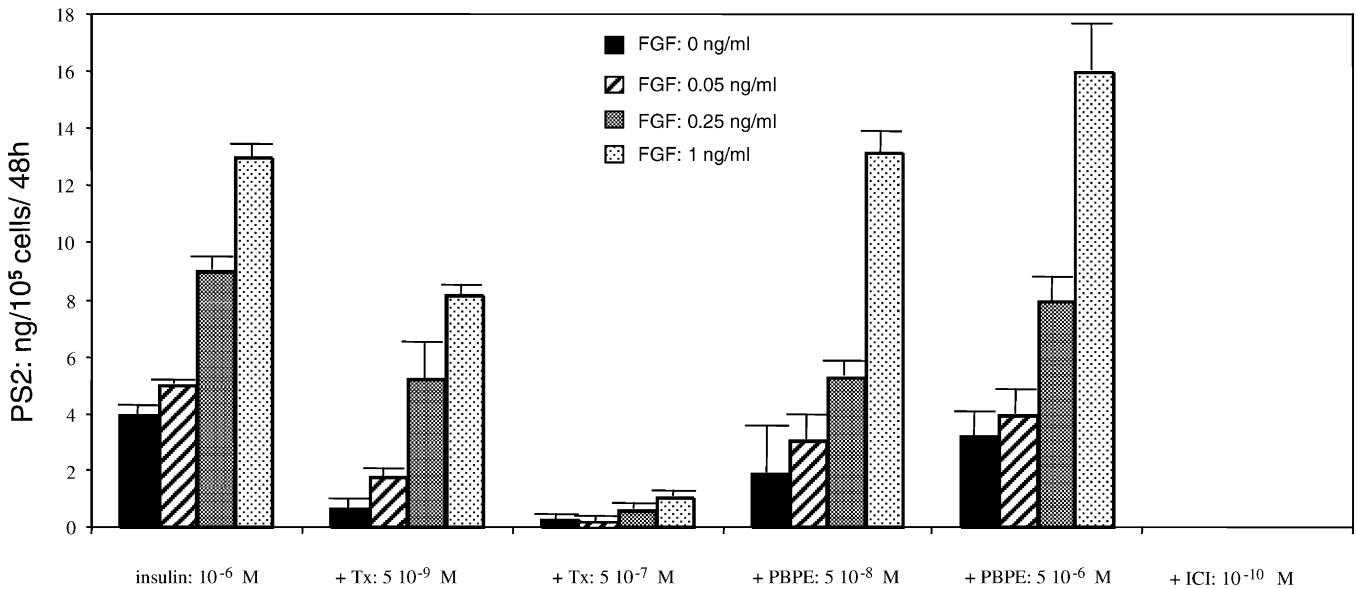

Fig. 8. Effects of anti-estrogens on pS2 secretion. Cells were grown in the presence of insulin 10^{-6} M, increasing concentrations of FGF-2 and anti-estrogens of the various families were added for 2 days. The cells were counted and pS2 was measured in the culture medium.

ER activation of the pS2 expression by insulin and FGF-2. Moreover it is shown that estradiol as FGF-2 and insulin induce separately a very low expression of pS2, their associations two by two give synergical effects.

4. Conclusions

The MCF-7 cell line has been widely used as a model for a better understanding of human breast cancer. Although several variations of this strain have been noticed depending on the laboratory culture conditions, many properties remain in common.

The breast cancer cell subline MCF-7^{SF} adapted to culture in chemically defined medium gave, with insulin example, a potential explanation for the controversy concerning the effects of FGF-2 on mammary tumor proliferation. On this cell subline the insulin activities; increase of, cell growth, PR and pS2 expressions are counteracted by anti-estrogens bound to ER suggesting a ligand independent activation of ER. This cell subline highlights the activities of anti-estrogens of various families. Each of the pure estrogen antagonist ICI 182,780, PBPE selective AEBS ligand and the equipotent ER and AEBS ligand tamoxifen, is able to decrease the proliferation rate in the absence of estrogen, they show a high efficacy to counteract the proliferative effect of, insulin, FGF-2, and E2. Moreover FGF-2 appeared as an activator of MCF-7^{SF} proliferation, acting synergistically with E2 whereas it served as an inhibitor, in the presence of insulin. These conflicting results fit with other unexplained observations reported with FGF-2 by Wang or Johnson, where FGF-2 is shown to induce both mitogenic and inhibitory G1 events [8,9]. Interestingly, although in the absence of insulin FGF-2 has the same effects as EGF on cell growth (i.e. increase of pS2, decrease of ER9 [30]), it decreases PR and does not appear to be a ligand independent activator of ER. However, potential crosstalk between FGF-2 and ER is evidenced by the synergistic effects of FGF-2 and E2 on cell growth and pS2 production, effects on pS2 which are reversed by pure anti-estrogen but not by selective AEBS ligand.

Acknowledgments

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