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Kinetics of Cellular Proliferation and Hormonal Receptors in EVSA-T Breast Cancer Cell Line

R. del Moral, J. C. Fernández, J. D. López-González, M. Gómez*, J. M. Ruiz de Almodóvar**, N. Olea and V. Pedraza

Departamento de Radiología
Facultal de Medicina
Universidad de Granada
18071 Granada (Spain)

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R. DEL MORAL, J. C. FERNÁNDEZ, J. D. LÓPEZ-GONZÁLEZ, M. GÓMEZ, J. M. RUIZ DE ALMODÓVAR, N. OLEA and V. PEDRAZA. *Kinetics of Cellular Proliferation and Hormonal Receptors in EVSA-T Breast Cancer Cell Line*. Rev. esp. Fisiol., 47 (1), 25-30, 1991.

The hormone-independent human breast cancer cell line EVSA-T, originally described as negative estrogen and progesterone receptors is shown to become positive hormone receptors when the cellular proliferation rate is slowed down. The experimental procedure included the following steps: 1) EVSA-T cells were seeded in minimum essential medium supplemented with 10 % fetal bovine serum and kept undisturbed for 2 days; 2) culture medium was replaced with Dulbecco's solution and Ham's F-12 and cells were incubated in serum-free media for another 24 h; 3) then, cells were «rescued» with 10 % FBS supplemented medium and estrogen (ER) and progesterone receptors (PgR) were measured immediately, time 0, and 6, 12, 18, 24 and 30 h after the media were changed. Cell yield was quantified at the same times. Experimental data indicate that changing the proliferation kinetics makes it possible to detect estradiol and progesterone receptors on EVSA-T cells. Estrogen receptor appeared at 18 h after rescue, 6 hours before progesterone receptor could be detected. Immunohistochemical analysis of ER content confirmed this observation, showing maximal positive stain at 18 h. Furthermore, ER disappeared when cells recovered their normal proliferation rate.

Key words: Cellular proliferation, Hormonal receptors, EVSA-T cell line.

Recent experimental data from several laboratories have shown that in breast tumors the expression of estrogen receptors (ER) and cell proliferation kinetics are strongly related (2, 10, 12). This observation has led to two important assumptions regarding tumor cell biology (19): a) tumors with an ER^+ phenotype should exhibit slow growth kinetics, and b) heterogeneity in ER phenotype among tumor cells may reflect the presence of cells with different growth rates. Furthermore,

* Departamento de Patología, Facultad de Medicina, Granada (Spain).
** To whom all correspondence should be addressed.

immunohistochemical techniques have revealed the complexity of ER expression patterns in both normal and cancerous breast tissue. Breast tumors showed a marked heterogeneity in antibody-receptor binding, together with the existence of ER^+ and ER^- cells (13). The biological significance of these findings has recently been reviewed (14). It has been suggested that the expression of a particular antigen may be related with the functional status of the tumor cells (7, 17, 18). This phenomenon could reflect the distribution of hormonedependent cells in the quiescent and proliferating compartments of cell cycle. In fact, the experiments performed with hormonedependent cells (19) have demonstrated that in the human breast cancer cell line MCF-7 hydroxytamoxifen exposure results in a blocking of cells in phases G_0/G_1 of cell cycle. A 3 to 4-fold increase in estrogen binding capacity has been observed when hydroxitamoxifen treated cells are compared to non-treated and mitotically active cells (4). The possibility that systemic treatment in breast cancer patients may lead to changes in the tumor ER phenotype has also been widely discussed (8, 15, 22). It seems that in breast tumors ER levels drop below the threshold for its detection in response to endocrine therapy with tamoxifen. Chemotherapy as well as hormonotherapy with compounds whose hormonal action differs from tamoxifen have occasionally resulted in the opposite effect, changing tumors initially characterized as ER^- into ER^+ (11, 21). Whether the receptor phenotype modification resulted from the pharmacologically induce deceleration of the tumor growth rate or from the selection of ER^+ cells, is still unclear and needs further investigation. This work was carried out to investigate the expression of estrogen receptors in the hormone-insensitive human breast cancer cells EVSA-T (11) when cultured in serum-free medium, strategy designed to decrease its growth rate.

Materials and Methods

Chemicals. — The following chemicals were used in all experiments: (a) [2,4,6,7-^{3}H]-17β-estradiol (Amersham, U. K.) with a specific activity between 85 and 110 Ci/mMol and a radioactive concentration of 1 mCi/ml; (b) 17α-methyl-^{3}H-promegestone (New England Nuclear, Boston, MA), with a specific activity between 77 and 101 Ci/mMol and a radioactive concentration of 1 mCi/ml; (c) 17β-estradiol (Sigma) and (d) 17α-methyl-promegestone (Russel-UCLAF, France).

EVSA-T cell line. — The EVSA-T cell line used in this study was first cultured in 1976 from a metastatic ascites fluid obtained from a patient with infiltrating ductal breast carcinoma (11). The presence of estrogen (ER) and progesterone receptor (PgR) was not initially reported. However, a recent report (6) showed that passage of EVSA-T cells from so-called «inactive» to «active» sera (5), resulted in the appearance of a progestin binding capacity in these cells. EVSA-T cells were kept in monolayer cultures in T-25 and T-75 flasks (Costar, Cambridge, MA) in a saturated humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. The culture medium was the minimal essential with Earle's salts (MEM) (Flow Laboratories, Irvine, Scotland) supplemented with 10 % heat inactivated (56 °C, 30 min) fetal bovine serum (FBS) (Flow Laboratories) and antibiotics (penicillin 100 UI/ml, streptomycin 100 µg/ml and gentamicin 40 µg/ml). Cell growth was estimated by DNA measurement using the method described by BURTON (1).

Estrogen and progesterone receptor measurements. — ER and PgR activities were measured using a whole cell assay that was previously described (16). To quantify ER and PgR receptors cultures were performed according to the following experimental procedure: 1) approx-

imately 100,000 cells were seeded in 35mm diameter Petri dishes (Costar) and cultured in 2 ml of MEM supplemented with 10 % FBS in a saturated atmosphere of 5 % CO_2 at 37 °C for 2 days; 2) culture media were discarded and replaced with 2 ml Dulbecco's medium + Ham's F-12 and cells kept in free-serum medium for 24 h; 3) cells were rescued with 2 ml MEM supplemented with 10 % FBS; 4) estrogen and progesterone receptors were assayed at 0, 6, 12, 18, 24 and 30 h after rescue. Cells were incubated for 1 h with 1 ml of nine different concentrations of 17β-estradiol and promegestone ranging from $3 \cdot 10^{-10}$ M to $7 \cdot 10^{-9}$ M, in the presence and absence of a 500-fold excess of cold competitor. The results of these assays were compared with the data collected from a group of cells cultured under the same experimental conditions except that they were not subjected to FBS depletion, and 5) cellular yields were quantified at each indicated time by measuring DNA content (1).

Immunohistochemical procedures. — Alternatively estrogen receptor immunodetection was performed at the times and the experimental conditions indicated for the whole cell assay. The immunostaining was performed according to the method described in the Abbott ER-ICA kit (Abbott Laboratories Diagnostics Division, North Chicago, IL). EVSA-T cells were cultured in petri dishes in cover-slips glass. Negative controls using the kit control serum and positive controls using MCF-7 estrogen-positive cells were routinely run.

Results

Proliferation yields of EVSA-T cells. — Figure 1 shows the growth data, expressed as μg DNA/well, of EVSA-T cells maintained for 24 h in serum-free medium Dulbecco's and Ham's F-12 media and

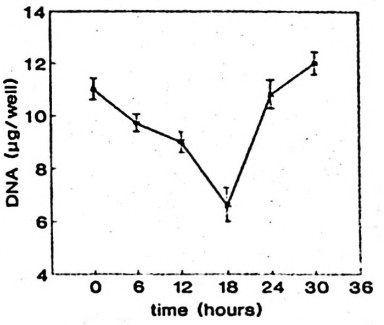

Fig. 1. *Growth of EVSA-T cells in 10 % FBS supplemented medium.*
Cells were maintained in culture dishes in serum-free medium for 24 h then media were changed to 10 % FBS supplemented medium (time = 0). Plates were harvested and DNA determined at the indicated times. Shown mean ± SE of quintuplicate dishes.

then rescued with MEM + 10 % FBS. For the first 18 hours following the change of the media, cell death was a prominent feature. From 0 to 18 h some cells recovered from blockage, repaired their lesions and entered the proliferative compartment. Moreover, from 18 to 30 h the amount of DNA in cultures doubled, indicating that those cells which survived FBS depletion entered and completed the DNA synthesis phase during this time.

Receptor Studies. — Maximal binding capacities for 17-β estradiol and progesterone in EVSA-T cells in relation to the post-rescue elapsed time are shown in figure 2. Cells were rescued with MEM supplemented with 10 % FBS and estrogen and progesterone receptors were quantified using an intact cell assay (18). Estrogen receptor was undetectable at 0, 6 and 12 h, became clearly positive at 18 and 24 h, and was absent once again by 30 h post rescue. Results indicated that cells depleted of FBS for 24 h decelerated its growth rate and resulted ER^+, while non serum depleted cultures were ER^-. Eighteen and twenty four hours after the se-

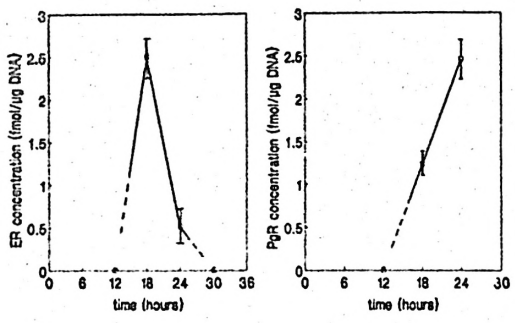

Fig. 2. *Estrogen and progesterone receptor concentrations in EVSA-T cells.*
Cells were maintained for 24 h in serum-free medium and then changed to 10 % FBS supplemented medium. At indicated time points after the change to serum supplemented medium, cells were incubated with (A) [^{3}H]17β-estradiol and (B) [^{3}H]promegestone with and without 200-fold excess of unlabeled steroids. The concentration of specific ER and PgR binding sites was determined by Scatchard plot analysis. Shown mean ± SE quintuplicate dishes.

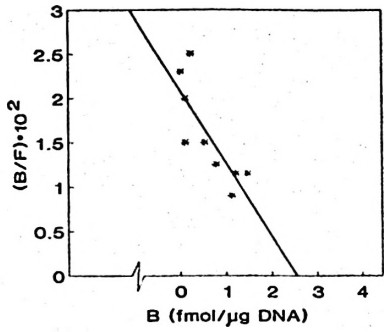

Fig. 3. *Scatchard plot of the specific binding of [^{3}H]17β-estradiol to EVSA-T cells at 18 h after rescue with serum supplemented medium.*
Cells were incubated for one hour with nine concentrations of [^{3}H]17β-estradiol ($3 \cdot 10^{-10}$ to $7 \cdot 10^{-9}$ M) with and without 200-fold excess of unlabeled 17β-estradiol.

rum supplemented medium was added, ER levels reached to 2.5 and 0.5 fmol/µg of DNA, with a ligand-hormone association constant of 6.5×10^{-9} M. Figure 3 shows the Scatchard transformation of the binding data for 17-β estradiol obtained at 18 h post rescue. Hill's transformation (3) of the 18 h binding capacity data (fig. 4) demonstrated the existence of a single protein-receptor binding site, or possibly multiple sites equivalent in terms of chemical binding characteristics. The linearity of the Hill plot together with the slope, $n_H = 1.00$ rule out the occurrence of cooperation phenomena which might have favored or hindered the hormonereceptor binding processes. No estrogen receptor could be detected by biochemical analysis in EVSA-T cells cultured in 10 % FBS supplemented MEM without previous passage in serum free medium (data not shown).

The results were confirmed by using a specific antibody against estrogen receptor. ER immunohistochemical evaluation revealed specific staining in the nuclei of EVSA-T cells at 18 and 24 h after rescue. The percentage of positive cells range from 56 % at 18 h to 10 % at 24 h.

Progesterone receptor expression lagged 6 h behind ER positive stain, a finding

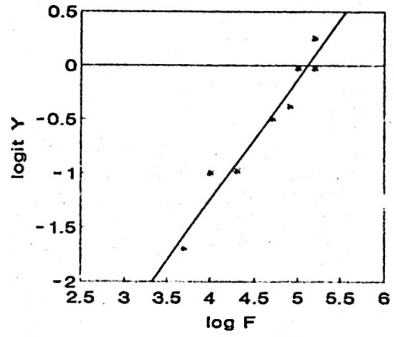

Fig. 4. *Hill's plot of [^{3}H]17β-estradiol binding data in EVSA-T cells at 18 h after rescue with serum supplemented medium.*
Logit Y is defined as (B/Bmax)/(1-B/Bmax). The experimental design is described in the legend to Fig. 3.

in accordance with current notions regarding PgR as a product of the action of estrogens in hormone sensitive cells. At 24 and 30 h PgR levels attained 1.3 and 2.5 fmol/μg DNA and the Kd obtained were 1.6×10^{-9} M, respectively.

Discussion

Control of cell proliferation by steroid hormones in estrogen sensitive tissues has been related for a long time with the existence of estrogen receptor but the estrogen action mechanisms on cell proliferation are badly understood (20). Previous studies showed that EVSA-T cells were devoid to sensitivity to estradiol (10^{-9} to 10^{-6} M) and the synthetic estrogen diethylstilbestrol (10^{-8} M) (9). The synthetic progestogen medroxiprogesterone acetate at 10^{-6} M remarkably produced a significant growth inhibition when these cells were cultured in some sera which endowed estradiol with growth promoting properties in MCF-7, ER, PgR positive breast cancer cells (5, 6). It was also shown that EVSA-T cells expressed progesterone receptor when maintained in that particular sera (6). The possibility to induce the appearance of PgR in EVSA-T cells in absence of ER was presented as a proof of a direct PgR inductive process independent of estrogen. The present paper shows that EVSA-T cells, considered for a long time as a genuine ER^- cell line, expressed estrogen receptor when the culture conditions were changed. Passage of cells from 10 % FBS supplemented medium to serum-free medium for 24 h and then «rescue» with 10 FBS supplemented medium, led to the demonstration of estrogen and progesterone receptors. The expression of the receptors is shown here to be time dependent, ER appeared and had a maximum at 18 h after rescue, was still positive at 24 h and fell to undetectable levels at 30 h. Progesterone receptor showed the same biphasic pattern but with a six hour delay, suggesting an expression estrogen-dependent.

Finally, whether the expression of estrogen and progesterone receptors in EVSA-T cells is a consequence of receptor positive clone selection or is related to cellular growth arrest in the cell cycle is now being explored.

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Resumen

Se demuestra que las células EVSA-T, línea celular hormono-independiente de cáncer de mama humano, originalmente descrita como receptor de estrógenos (ER) y progesterona (PgR) negativa, pueden expresar ER cuando el ritmo de proliferación se enlentece. El procedimiento experimental consta de las siguientes etapas: 1) Siembra de células EVSA-T en medio esencial mínimo (MEM) suplementado con 10 % de suero bovino fetal (FBS), mantenidas dos días en estas condiciones; 2) Reemplazo del medio de cultivo por medio Dulbecco y Ham F12 e incubación de las células en medio libre de suero durante 24 h; 3) Rescate posterior de las células con MEM más 10 % de FBS y medida inmediata (tiempo 0) de la concentración de ER y PgR, y a las 6, 12, 18, 24 y 30 h después del cambio de medio. El número de células presentes en el cultivo se cuantifica a esos mismos tiempos. Los datos experimentales indican que el cambio de la cinética de proliferación hace posible detectar ER y PgR en las células EVSA-T. Las células ER positivas se encuentran a las 18 horas del rescate, seis horas antes de la detección de positividad de PgR. El análisis inmunohistoquímico de ER confirma

esta observación mostrando la máxima fijación a las 18 horas. Posteriormente, cuando las células recobran su ritmo normal de crecimiento, el ER vuelve a ser indetectable.

Palabras clave: Proliferación celular, Receptores hormonales, Línea celular EVSA-T.

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