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STABLE TRANSFECTION OF THE ESTROGEN RECEPTOR cDNA INTO Hela CELLS INDUCES ESTROGEN RESPONSIVENESS OF ENDOGENOUS CATHEPSIN D GENE BUT NOT OF CELL GROWTH

Isabelle TOUITOU, Marc MATHIEU and Henri ROCHEFORT

INSERM U 148
Unit Hormones and Cancer
60 Rue de Navacelles
34090 Montpellier,France

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Cathepsin D, a lysosomal protease, is induced by estrogens in hormone responsive breast cancer cells, by progesterone in normal endometrium and expressed at high constitutive levels in estrogen receptor (ER)-negative cells. To investigate whether ER is the only transacting factor missing in ER negative cells to obtain estrogen regulation, we transfected an ER cDNA expression vector (HEO) into ER-negative Hela cells and showed that it could recover estrogen sensitivity for cathepsin D gene expression but not for cell growth regulation. These results show i. that the expression of an endogenous gene in humans can be stimulated by estradiol after ER supplementation, indicating that Hela cells contain sufficient amounts of the other transcription factors required for cathepsin D estrogen inducibility ; ii. that cathepsin D expression is stimulated by estrogens in this cervix cancer cell line, as it is in the ER-positive breast cancer cells, which contrasts with its regulation by progesterone in normal endometrial cells.

Estrogens stimulate the expression of several genes via binding to and activation of the estrogen receptor (ER), behaving as a ligand activated transacting transcription factor (1). In well differentiated ER-positive breast cancer cells, estrogen also stimulates cell growth both *in vivo* and *in vitro* (2). However, half of these ER-positive breast cancer cells are unresponsive to estrogens and the mechanism by which these cancer cells become unresponsive is not yet understood (3). Some growth factors (4) and cathepsin D (cath-D), a lysosomal protease (5), are secreted and inducible by estrogens and are thought to play a role in mediating the effect of estrogens on cell growth and invasion. High levels of cath-D mRNA and protein are also present in hormone independent breast cancer cells but not regulated by estrogens. Moreover, the hormonal regulation of cath-D appears to be tissue specific, since its gene is inducible by progesterone but not

ABBREVIATIONS
E2: 17 β estradiol, ER: estrogen receptor ; PR: progesterone receptor ; CAT: chloramphenicol acetyl transferase ; DMEM: Dulbecco's modified eagle's medium ; FCS: fetal calf serum ; cDNA: complementary deoxyribonucleotide acid ; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis ; Cath-D: cathepsin D.

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by estrogens in rat and human normal endometrial cells (6,7). The Hela cell line, derived from uterine cervix cancer, produces constitutively high levels of cath-D, and represents an estrogen-resistant cancer model expressing no ER sites. Since the normal uterine cervix contains ER and responds to estrogen (8), we transfected the HEO ER expressing vector (9) into Hela cells in an attempt to recover estrogen responsiveness for cath-D expression and cell growth.

MATERIALS AND METHODS

CELL CULTURE AND GROWTH

Hela cells and MCF7 cells were cultured in DMEM supplemented with 5% and 10% foetal calf serum (FCS) respectively. The culture conditions for studying the effect of E2 and DNA assay for growth evaluation were performed as described previously (10).

TRANSFECTION OF THE ER-cDNA AND SCREENING OF ER-POSITIVE CLONES

Half confluent cultures of Hela cells were co-transfected with 10 µg of HEO and 100 ng of PY3 (11) (expression vector for the hygromycine resistance gene), using the calcium phosphate technique (12). Stable cell lines surviving in 200 µg/ml hygromycine (Sigma, France) were expanded, withdrawn and transiently transfected with 200 ng Vit-tk-CAT (13), in the presence or absence of 10 nM E2. CAT assays were performed as described previously (14).

LABELING, IMMUNODETECTION AND ELECTROPHORESIS OF CATH-D

After stimulation by E2, the cells were labeled for 1 or 6 hours with 200 µCi/ml of L^{35}S methionine (Amersham, England) in DMEM containing 10% of the normal methionine concentration. Pulse chase experiments were performed as described previously (15). The amounts of secreted and cellular labeled proteins were determined by precipitation with trichloracetic acid (16) before solid phase immunodetection (17) with the D7E3 monoclonal antibody (18).

The samples were analyzed by sodium dodecyl sulfate-gel electrophoresis and processed for fluorography as described previously (17). Fluorographs were scanned using a scanning densitometer (Joyce Loebl, England).

RESULTS

EXPRESSION OF ER AND CATH-D IN TRANSFECTED Hela CELLS

The expression plasmid HEO containing the full-length estrogen receptor cDNA isolated from MCF7 cells was transiently transfected into Hela cells. Cath-D levels, as measured by Elisa (19), were increased by 2- to 4-fold in the cell extracts and conditioned media following transient transfection by the calcium phosphate technique, due to the high calcium concentration used (12 mM) (I. Touitou, unpublished observation). No E2 effect (10 nM) was observed under these conditions.

We therefore decided to isolate ER expressing cloned Hela cells. A stable transformant Hela 4 (RE) was selected for its ability to induce Vit-tk-CAT, a transiently transfected E2 reporter gene, in the presence of E2 (not shown). The expression of ER in Hela 4 (RE) cells was confirmed by immunocytochemistry and by measuring the saturable uptake of ^{3}H estradiol into these cells, resulting in an estimated ER concentration of 5-10 fmoles/mg cytosol proteins. Southern blot

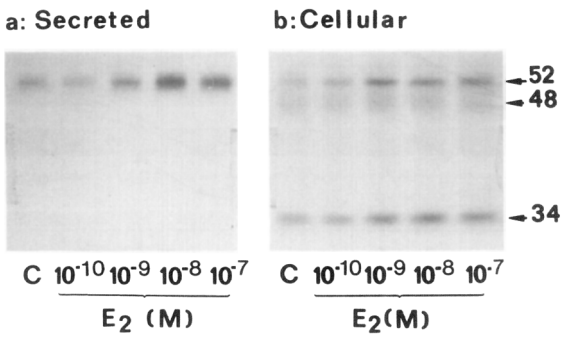


Figure 1
EFFECT OF E2 IN Hela 4 (RE) CELLS ON SECRETED (a) AND CELLULAR (b) CATH-D ANALYZED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Hela 4 (RE) cells were withdrawn for 5 days then, vehicle alone (C), or increasing concentrations of E2 were added into the medium as indicated. Cells were labeled with ^{35}S Methionine as described in Materials and Methods. Secretion media and cell extracts containing the same amount of TCA precipitable counts (5.10^4cpm and 10^6cpm respectively) were immunodetected with the D7E3 monoclonal antibody and then analyzed by SDS-PAGE.

analysis of Xba1-digested high molecular weight DNA of Hela 4 (RE) cells indicated that the transfected ER cDNA was correctly integrated (not shown).

EFFECTS OF ESTRADIOL ON CATH-D EXPRESSION IN ER-POSITIVE Hela 4 (RE) CELLS

As shown in Figs. 1 and 2, E2 treatment of Hela 4 (RE) cells resulted in 2- and 4-fold increases in the levels of cellular and secreted cath-D respectively, but did not modify its basal expression in Hela 32 (V) cells transfected with the pSG1 vector alone (Fig. 2c,d). The stimulatory effect of E2 on Hela 4 (RE) cells (Figs. 1 and 2a,b) was lower than that observed in MCF7 cells (Fig. 2e,f), and the maximal increase occurred at a concentration of 10 nM, compared to 0.1 nM for MCF7 cells. This discrepancy can now be explained by the fact that HEO ER has a 100 fold-lower affinity than wild type ER due to an artefactual amino acid change in position 400 (20). Similar increased secretion of cath-D following E2 treatment was determined by immunoradiometric assay (21) of cath-D which had accumulated for three days in conditioned media (not shown). The increased synthesis of cath-D by E2 was probably transcriptional since it was preceded by an accumulation of its 2.2 kb mRNA, analyzed by Northern blot (6) (not shown). However, the proportion of cath-D cellular forms was not the same in Hela 4 (RE) and in MCF7 cells, suggesting different intracellular cath-D processing in these cells. The E2-induced increase was especially found for the pro-enzyme (52K) and the final mature chain (34K), whereas it was lower than in MCF7 cells for the intermediate form (48K) (Fig. 2b).

In order to study the processing of cath-D in Hela 4 (RE) cells, we performed pulse chase experiments which confirmed that the 3 forms of cath-D (52K, 48K and 34K) were increased by E2, while no modifications were observed in the

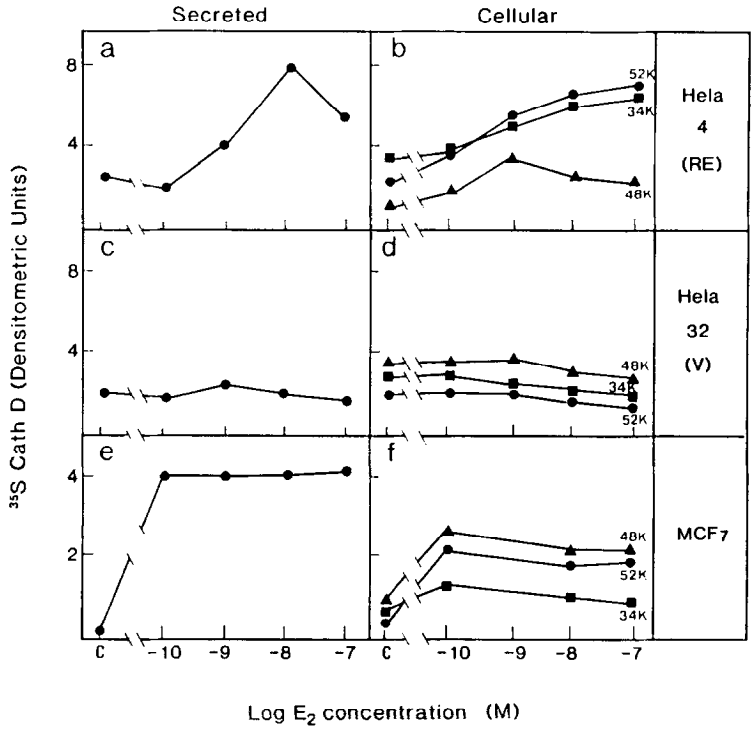


Figure 2
DENSITOMETER SCANS OF ^{35}S LABELED IMMUNOREACTIVE CELLULAR AND SECRETED PROTEINS SYNTHESIZED AND RELEASED BY CELLS FOLLOWING E2 TREATMENT

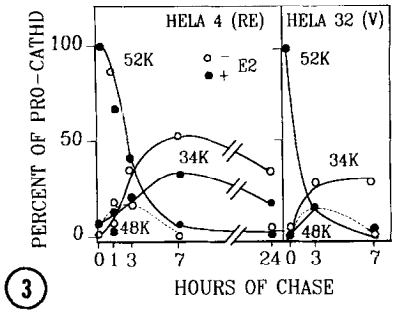
Relative amounts of ^{35}S labeled secreted or cellular cath-D measured by scanning the autoradiographs shown in Fig. 1 (a,b), or in parallel experiments performed on Hela 32 (V) cells (c,d) and MCF7 cells (e,f). The three cellular forms of cath-D (52K o, 48K▲, 34K■) were quantified separately.

control clone Hela 32 (V). Moreover, as shown in Fig. 3, there was no stabilization of pro-cath-D by E2, in contrast to what was previously described in MCF7 cells (22). The ratio of the different forms of the cellular enzyme following the chase was the same in the presence or the absence of E2, except for the 34K form which was lower due to the increased secretion of the immature form.

We conclude that E2 is able to increase the expression and secretion of pro-cath-D in Hela cells transfected with the ER cDNA without altering its intracellular processing.

EFFECTS OF ESTRADIOL ON GROWTH OF HELA 4 (RE) CELL LINE

The growth rate of Hela cells remained unchanged following transfection of the ER cDNA. In contrast to the stimulatory effect of E2 on cath-D expression and on growth of MCF7 cells, the growth of the ER expressing Hela cells was not affected by E2 at any concentration (Fig. 4a) or treatment time (Fig. 4b). The same unresponsiveness to 10nM E2 was found by measuring the tritiated thymidine uptake and cell cloning in soft agar (not shown).



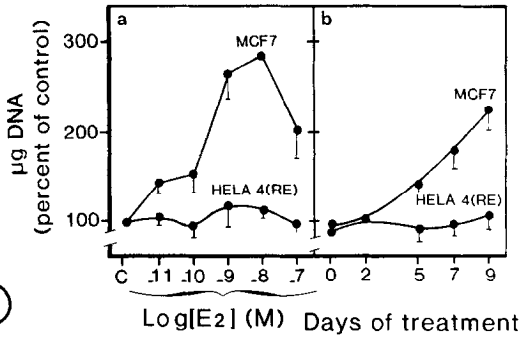


Figure 3

PROCESSING OF PRO-CATH-D IN THE ER-POSITIVE (Hela 4 (RE)) AND ER-NEGATIVE (Hela 32 (V)) TRANSFECTED CELL LINES USING PULSE CHASE EXPERIMENTS

Withdrawn Hela 4 (RE) and Hela 32 (V) cells were treated for 48 hours with 10 nM E2 (solid circles) or vehicle alone (open circles), then labeled for 1 hour with ^{35}S Methionine and chased for the indicated periods of time with unlabeled methionine. The cell lysates, containing 10^6 cpm of TCA precipitable proteins, were immunodetected and submitted to SDS-PAGE. Quantification of the different forms (pro-cath-D 52K ; intermediate 48K and mature 34K) was carried out by scanning the fluorograph. Results are expressed in percent of pro-cath-D at time 0.

Figure 4

ABSENCE OF EFFECT OF E2 ON THE GROWTH OF Hela 4 (RE) CELLS COMPARED TO THAT OF MCF7 CELLS

MCF7 and Hela 4 (RE) cells stripped of endogenous steroids were plated at a density of 15,000 cells per well in 24-well dishes in culture medium containing 3% charcoal treated FCS. Two days later, they were treated with 1% charcoal treated FCS for (a) 7 days with increasing concentrations of E2 or vehicle alone (C) or for (b) increasing periods of time with 10 nM E2 or vehicle alone. At the end of the treatment, growth of the cells was evaluated as indicated in Methods. Results are expressed in percent of control.

DISCUSSION

The constitutive expression of cath-D in Hela cells is similar to that of MCF7 cells and secretion levels are 2- to 4-fold lower. This amount was only slightly raised by the stable transfection. The introduction of human estrogen receptor cDNA alone into the ER-negative Hela uterine cervix cancer cells was sufficient to stimulate the synthesis of endogeneous cath-D when cells were treated by estradiol. These results show that ER is the only missing trans-acting factor in Hela cells, which is able to confer an E2 sensitivity to cath-D even at low ER expression levels. They also suggest that in uterine cervix cancer cells, cath-D gene is potentially inducible by estrogen, whereas it is not in endometrial cells (6,7). By contrast, the addition of a functional estrogen receptor was not sufficient for triggering the expression of other genes (progesterone receptor and pS2) which are estrogen inducible in breast cancer cells (our unpublished data).

The lack of E2-induced growth stimulation of the transfected cells may be due to i) a constitutive high level production of mitogens which by-pass the estrogen-regulated cell growth pathway ; ii) the absence of other factors required for cell growth regulation but not for cath-D gene regulation or iii) the low expression of ER in Hela 4 (RE) cells, since it has been shown that intracellular

receptor concentrations limit glucocorticoid-dependent enhancer activity (23). However, similar dissociation between gene regulation and growth response to steroids was also observed in S115 mouse mammary cells (24).

The exogenous estrogen-responsive promoter recombinants Vit-tk-CAT and ps2 CAT have been found to respond to E2 following transfection of the ER cDNA into MCF7 (13), Hela cells (25) and Hela 4 (RE) clone (Touitou, unpublished data) contrasting with the few examples of endogeneous genes which can be regulated following transfection of a steroid receptor. The tyrosine aminotransferase gene of rat hepatoma cells responds to the transfected glucocorticoid receptor (23) or progesterone receptor (26) whereas no estrogen-sensitive endogeneous gene was identified in an ER-supplemented human osteoblastoma cell line (27). We describe here, for the first time, that an endogenous E2-responsive gene is regulated in human cancer cells after ER stable transfection. The Hela 4 (RE) cell line appears therefore a good model for studying the estrogen regulation of cath-D and possibly other induced proteins.

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