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HORMONAL REGULATION OF CATHEPSIN D FOLLOWING TRANSFECTION OF THE ESTROGEN OR PROGESTERONE RECEPTOR INTO THREE SEX STEROID HORMONE RESISTANT CANCER CELL LINES

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Summary—The cathepsin D gene is differentially regulated by estrogens in hormone responsive breast cancer cells, by progestins in normal human endometrium and is highly expressed but not regulated by these steroids in estrogen (RE)- and progesterone receptor (RP)-negative breast cancer cells. We have stably transfected the RE-negative breast cancer cell line MDA-MB 231 and the Hela cell line with an expression vector for the human RE. The endogenous cathepsin D which is constitutively expressed was further stimulated by estradiol. However, the growth of both cell lines was not stimulated by estradiol and could not be inhibited by the antiestrogen ICI 164,384. By contrast, the cathepsin D gene in the estrogen responsive Ishikawa endometrial cancer cell line was unresponsive to estrogen or to progesterone even following stable transfection of expression vectors for the RP (both A and B isoforms). We conclude that the cathepsin D gene is potentially responsive to estrogens in MDA-MB 231 and Hela cells, which therefore express all of the transcriptional machinery (except the RE) necessary for this regulation. By contrast, cathepsin D remains unresponsive to estrogen and progesterone in Ishikawa cells. The cathepsin D gene is one of the first examples of an endogenous steroid responsive gene which can be controlled by steroids following stable transfection of a steroid receptor.

INTRODUCTION

Cathepsin D (cath-D) is a lysosomal aspartyl proteinase that is expressed at a low level in all cells, but overexpressed in breast cancer cells. Overexpression of this protease is followed by secretion of the pro-enzyme and is associated with an increased risk of developing metastases (for review see [1]). The mechanism of hormonal regulation of the cath-D gene is complex, involving both steroid and growth factors [2], and varies according to the target cells studied. In estrogen receptor (RE)-positive breast cancer cells, cath-D mRNA is induced by estrogens but not by progestin or other steroid hormones. In RE-negative breast cancer and Hela cells, cath-D expression is no longer regulated by estrogens but levels are still higher than in normal mammary cells.

In normal endometrium, cath-D protein and mRNA [3] is induced by progesterone but not by estrogen [3, 4]. A study of tissue specific-regulation of cath-D in different sex steroid target tissues may improve our understanding of the mechanisms involved in hormonal regulation of gene expression and hormonal carcinogenesis in eucaryotic cells. An early modification of the hormonal regulation of this gene, that codes for a protease, may occur during the initial steps of carcinogenesis. In order to determine whether this differential regulation by sex steroid hormones is due to tumor progression (normal cells vs cancer cells) or to tissue specificity (endometrium vs mammary cells), we studied cath-D gene regulation in the Ishikjawa endometrial cancer cell line which contains both RE and progesterone receptors (RP).

Conversely, during tumor progression, hormone independency sometimes occurs with loss of the RE [5] but this is not always the case. It might therefore be interesting to determine whether regulation of the cath-D gene is also modified in hormone independent cancer cells following transfection of the RE. Finally, antiestrogens specifically inhibit the growth of 50 to 60% of RE-positive breast cancer tumors, but are generally inefficient on growth-inhibition of RE-negative breast cancer cells. It has been proposed that antiestrogens might inhibit both estrogen and growth factor activities via the

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RE [6, 7]. It would therefore be of considerable therapeutical interest to recover hormonal sensitivity to antiestrogens by transfecting the RE gene into RE-negative cancer cells. We transfected the RE gene expression vector into MDA-MB 231 and Hela cells, two RE-negative cell lines, in an attempt to recover their hormonal sensitivity for both cath-D gene expression and cell growth control.

We review here the differential regulation of the cath-D gene in uterine and mammary cells and report for the first time on estrogen regulation of cath-D in the MDA-MB 231 breast cancer cell line following transfection by the RE expression vector.

EXPERIMENTAL

Cell culture and hormonal treatment

Hela, MCF7, MDA-MB231 and Ishikawa cells were cultured in DMEM supplemented with 5, 10, 10 and 15% fetal calf serum (FCS), respectively. Estradiol (E2) and promegestone (R5020) were provided by Roussel Uclaf (Romainville, France) and 4-hydroxy tamoxifen (4 OHT) and ICI 164,384 were provided by ICI (Macclesfield, U.K.). All hormones were added to the culture medium at a final ethanol concentration of 0.1%.

Prior to each hormonal treatment, the cells were placed for one week in phenol red free DMEM (unless specified) containing dextran-coated charcoal-treated serum (FCS/DCC).

Transfection experiments

Cells were transfected at 30–50% confluency with CAT (chloramphenicol acetyl-transferase) reporter genes using the calcium phosphate technique [8]. Transiently transfected cells were treated with hormones, cell extracts were prepared and CAT assays were performed as described previously [3, 9].

The Hela RE-positive clone [9] was transfected with the HEO [10] expression vector containing human mutated RE cDNA inserted into the pSG_1 plasmid. The MDA RE-positive clones were stably transfected with the HEGO expression vector containing normal RE cDNA [11] as described separately (F. Vignon *et al.*, in preparation).

Ishikawa RP-positive clones were selected and screened as follows: Cells were plated into 6-well dishes, cotransfected with 5 μg expression vectors for form A, B or A and B RP genes (PR) (hPR1: form B, hPR2: form A) [12] and 250 ng expression vector for the hygromycine B resistance gene PY3 [13]. Clones surviving in 200 μg/ml hygromycine (Sigma, France) were expanded and screened for their ability to induce "LTR-tk-CAT" [14], a transiently transfected PR gene in the presence of 1 nM R5020 following one week hormonal deprivation to suppress the endogenous RP which is totally estrogen-dependent in this cell variant [3].

Immunodetection of cath-D

Two complementary methods were used to quantify cath-D:

(i) Solid phase immunodetection following [^{35}S]methionine labeling of cellular and secreted proteins was carried out as described previously [15], except that 24-multiwell dishes were used for labeling and that the amounts of secreted and cellular labeled proteins were determined by precipitation with TCA prior to immunodetection in order to standardize the assay.

(ii) An immuno radio metric assay (IRMA). Steroid withdrawn cells were treated for 2 to 4 days with the appropriate hormone. Secreted and cellular proteins were prepared, and cath-D was quantified using a "ELSA cath-D" kit (Biotechnics, France) as described previously [16].

RESULTS

Induction of cath-D by E2 following transfection of the RE into Hela cells

Hela cells which derive from a carcinoma of the uterine cervix, contain no RE or RPs, however they constitutively produce a similar quantity of cath-D to that of MCF7 cells and secrete 20–50% of pro-cath-D. To determine whether the endogenous cath-D gene is potentially regulated by estrogen or progesterone, we transfected the human RE gene (HEO) [10] into these cells and selected a stable transfectant named Hela 4 (RE).

Both secreted and cellular cath-D were induced [9] when Hela 4 (RE) cells were treated with increasing concentrations of E2. Cath-D was increased 2- to 4-fold [Fig. 1(a) and (b) and Fig. 2(a)] as is the case in MCF7 breast cancer cells [Fig. 1(e) and (f)].

However, higher concentrations of E2 were required to induce cath-D, which can now be explained by the fact that the transfected RE is

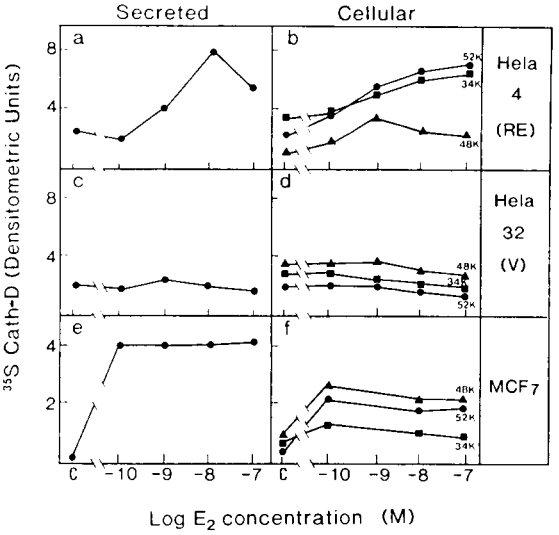

Fig. 1. Densitometer scans of ^{35}S labeled immunoreactive cellular and secreted proteins synthesized and released by cells following E2 treatment. Hela and MCF7 cells were withdrawn for 5 days then, vehicle alone (C), or increasing concentrations of E2 were added into the medium as indicated. Cells 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 (5×10^4 cpm and 10^6 cpm, respectively) were immunodetected with the D7E3 monoclonal antibody and then analyzed by SDS–PAGE. Relative amounts of ^{35}S labeled secreted or cellular cath-D measured by scanning the autoradiography are shown. The three cellular forms of cath-D (52 ●, 48 ▲ and 34 K ■) were quantified separately. Reproduced by permission from Ref. [9].

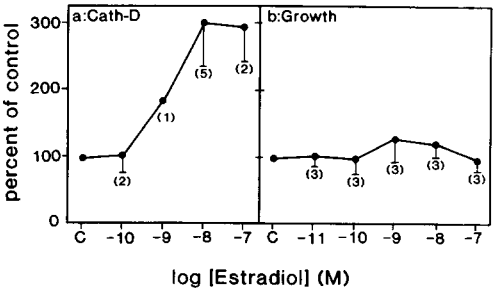

Fig. 2. Effect of E2 on cath-D gene expression (a) and cell growth (b) in Hela 4 (RE) cells. (a) Hela 4 (RE) cells were deprived of steroid hormones for 1 week and treated for 2–4 days with the indicated E2 concentrations. Cells were then labeled 6 h with [^{35}S] methionin. Secretion media were immunoprecipitated and analyzed by 12% SDS–PAGE. Fluorographs were read on a densitometer chromoscan (Joyce-Loebl, U.K.) and arbitrary units were deduced from the traces. The mean of different experiments (number in brackets) + SD are represented. (b) 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% FCS/DCC. Two days later, they were treated with 1% FCS/DCC for 7 days with increasing concentrations of E2 or vehicle alone (C). Growth of the cells was evaluated by assaying DNA [9].

artefactually mutated in the hormone binding domain, thus resulting in decreased affinity of the estrogen for its receptor [11].

By contrast, cells transfected with the pSG_1 vector alone, without the RE sequence (Hela 32-V) [Fig. 1(c) and (d)], remained unresponsive to E2. However, the relative proportion of the 3 cellular forms differed in Hela cells (more 34K mature form) compared to MCF7 cells (more 48K intermediate form) and the intracellular pro-enzyme half-life was not stabilized by E2 as it is in MCF7 cells [9]. Cell growth was not stimulated by E2 in the Hela 4 (RE) clone at any E2 concentration [Fig. 2(b)]. The 4 OHT and ICI 184,384 antiestrogens were also inefficient for cell growth inhibition (not shown).

Effect of E2 and antiestrogens on cath-D gene expression and cell growth following transfection of the RE cDNA into MDA-MB 231 breast cancer cells

To investigate whether the progression of cath-D towards hormono-independency was only due to the loss of RE, we studied the effects of E2 and antiestrogens on the MDA-MB 231 RE-negative cell line. This cell line was stably transfected with the HEGO expression vector containing normal human RE cDNA [10]. Several stable clones were obtained (F. Vignon *et al.*, in preparation). Both secreted and cellular cath-D were stimulated 3- to 5-fold following 10 nM E2 treatment of the MDA-HC_1-positive clone which contains 2 fmol RE/μg DNA (Fig. 3). The dose response curve was similar to that obtained with MCF7 cells (Fig. 4). 10 pM to 0.1 nM E2 was enough to increase cath-D secretion, with a maximal increase being obtained at 1 nM E2. A 2-fold stimulation of cath-D secretion was also obtained with the MDA-HE5 RE-positive clone which contains 2.2 fmol RE/μg DNA (not shown). By contrast, E2 was without effect on another MDA-RE-positive clone. (MDA-HD1: 1.6 fmol/μg DNA) and on a MDA RE-negative control clone which had been transfected with the pSG_1 vector alone (pB5) (not shown). The pure antiestrogen ICI 164,384 (1 μM) (Fig. 3 lanes 5 and 10) antagonized induction of cath-D by E2. When another antiestrogen, 4 OHT, was used alone, it did not display any agonist activity (Fig. 4). While the growth of MCF7 cells was inhibited by ICI 164,384 and rescued by the addition of 0.1 μM E2, indicating an RE-mediated growth inhibition, this antiestrogen was

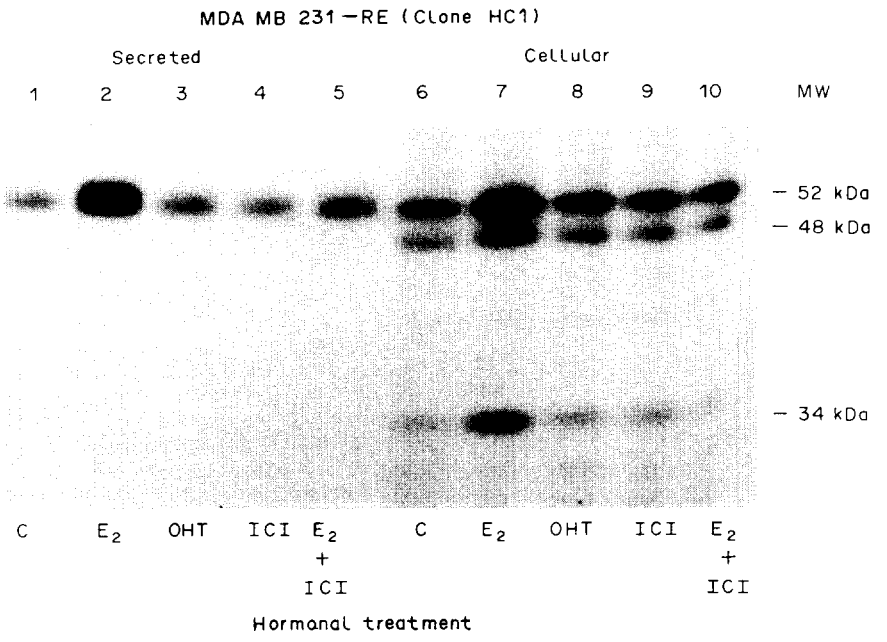

Fig. 3. Effects of E2 and antiestrogens on a RE-transfected clone MDA-MB 231. MDA RE-positive cells (clone HCl) were grown for 1 week in DMEM supplemented with 10% FCS/DCC, then treated for two days with: ethanol 1‰. (1, 6), 10 nME2 (2, 7), 1 nM 4 OHT (3, 8), 1 nM ICI 164,384 (4, 9) and 10 nME2 + 1 μM ICI 164,384 (5, 10). Cells were then labeled, and cell extracts and secretion media were analyzed by SDS–PAGE as described in Fig. 2. A typical fluorograph is shown.

totally inefficient on growth inhibition of two RE transfected MDA-MB 231 clones (Fig. 5). E2 alone also had no effect (not shown). The slightly decreased growth noted for MDA-HE5 and PB5 clones was probably due to a cytotoxic effect of this antiestrogen at a 1 μM concentration, since it was not rescued by 0.1 μM E2. Thus MDA-HCl and MDA-HE5 RE-positive clones behaved as MCF7 cells for cath-D gene regulation but remained unsensitive to hormonal or antihormonal treatment for cell growth.

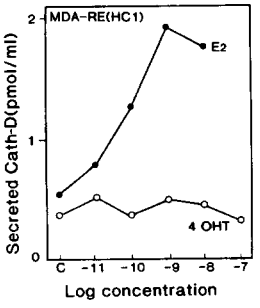

Fig. 4. Level of cath-D secreted by MDA RE-positive cells (HCl clone) following treatment by E2 or 4 OHT. Cells were withdrawn from exogenous steroids as described in Fig. 3 and treated with ethanol 1‰. (C) or with the indicated concentrations of E2 (●) or 4 OHT (○) for 3 days. DMEM containing phenol red was used for 4 OHT treatment. Secreted cath-D was then directly quantified by immunoradiometric assay of the conditioned medium.

Regulation of the cath-D gene in human endometrium

Cath-D is under progesterone control in normal endometrial cells, whereas it is regulated by E2 in breast and Hela RE-positive cancer cells. In women, the level of cath-D is higher in

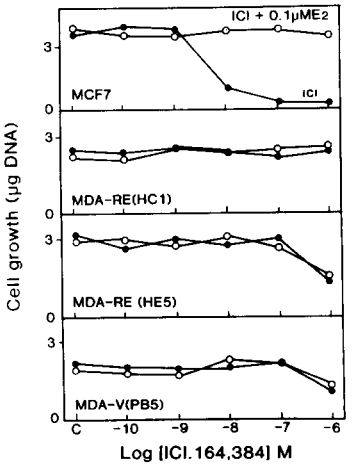

Fig. 5. Absence of response of two MDA RE-transfected clones to the antiestrogen ICI 164,384. Cells were withdrawn for 1 week from exogenous steroids as follows: they were kept in DMEM (without phenol red) supplemented with 10% FCS/DCC for two days, then 3% FCS/DCC for another 48 h, then seeded into 24-well dishes. Two days later they were placed in medium containing phenol red and treated with the indicated ICI 164, 384 concentrations in the absence (●) or presence (○) of 0.1 μM E2.

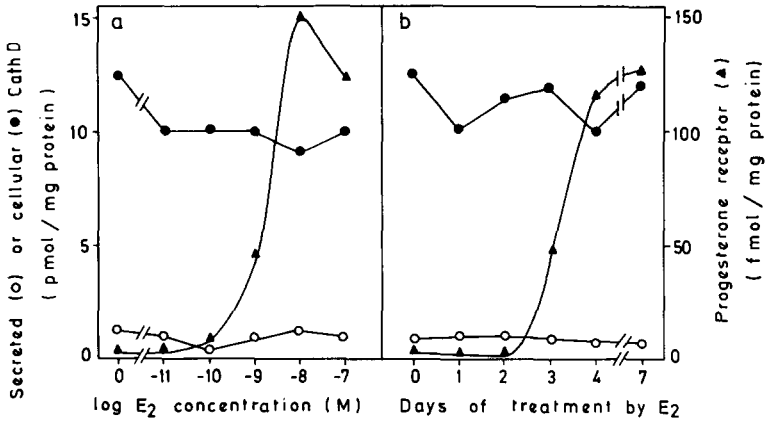

Fig. 6. Effects of E2 on cath-D and RP in Ishikawa cells. Two typical experiments are represented which were repeated at least once. (a) Dose-response of Ishikawa cells to E2: Ishikawa cells were plated in Petri dishes, E2 was added to the medium at the indicated concentrations, and culturing was carried out for 4 days. The conditioned media were then removed from the cells and assayed for secreted cath-D. Cell extracts were prepared from each plate and used for both cellular cath-D and RP determinations. All values were corrected according to the corresponding protein contents of the cell extracts. (b) Time course of E2 effects: Ishikawa cells were plated and maintained with FCS/DCC, at least 4 days prior to treatment as in (a). E2 (10 nM) was then added to each plate for the indicated times. All cells were harvested 11 days after seeding and cath-D or RPs were assayed. Reproduced by permission from Ref. [3].

the luteal phase than in the follicular phase and is induced in epithelial endometrial cells previously treated in primary culture with R5020 [3, 4, 17].

We wanted to investigate whether this differential hormonal regulation was specific to endometrial tissue, or specific to normal cells compared to cancer cells. We thus studied the effect of estrogen and progestins on the endogenous cath-D gene expressed in a well differentiated endometrial cancer cell line (Ishikawa) which contains both RE and RP. Despite the presence of functional RE in this cell line, as shown by the estrogen induction of a transfected E2 reporter gene (Vit-tk-CAT) [18] and of the endogenous RP, levels of the secreted and cellular protein and mRNA cath-D were unaffected by E2 (Fig. 6). The fatty acid synthetase (C. Joyeux, unpublished results) and the cath-D protein and mRNA [3] were also not induced by the progestin R5020. However, the transfected progesterone reporter gene LTR-tk-CAT [13] was greatly induced by this hormone when Ishikawa cells were pretreated with 10 nM E2 to be able to obtain a sufficient endogenous RP expression in these cells [3]. This conflicting result led us to suspect that the endogenous RP could be deleted or mutated since in Hela cells for instance, hormonal induction requires the N-terminal sequence of the RE for the PS2-CAT, but not the Vit-tk-CAT E2 reporter genes [19, 20]. An alternative possibility could be the absence of one of the two isoforms (A or B) of the RP or of some other transactivating factors. We therefore stably transfected the expression vectors for the RP form A (hPR1) and/or form B (hPR2) [12] into Ishikawa cells. To validate our screening test, we controlled that no induction of the transiently transfected LTR-tk-CAT could be obtained in the absence of E2 pretreatment in Ishikawa wild type cells [Fig. 7(a)] and also that cotransfection of exogenous RP was required in these conditions [Fig. 7(b)]. We used these criteria to specifically screen the exogenous RP expressing clones following stable transfection of this receptor. No progestin induction of secreted cath-D could be shown for any of the 13 clones expressing form A, B or A and B RP which were studied. Figure 8 represents a dose response curve for 7 of these clones containing only transfected form A [Fig. 8(a)], form B [Fig. 8(b)] or both forms [Fig. 8(c)] of the RP. The same unresponsiveness of cellular cath-D to 1 nM R5020 was found in all cases (not shown).

We conclude that the reason for the possible unresponsiveness of endogenous cath-D to progestin is not located at the RP level but rather at another one (lack of specific transcription factors or altered regulatory sequences of the cath-D gene).

DISCUSSION

The transfection of REs and RPs into cell lines has allowed us to answer a few questions concerning regulation of the cath-D gene and

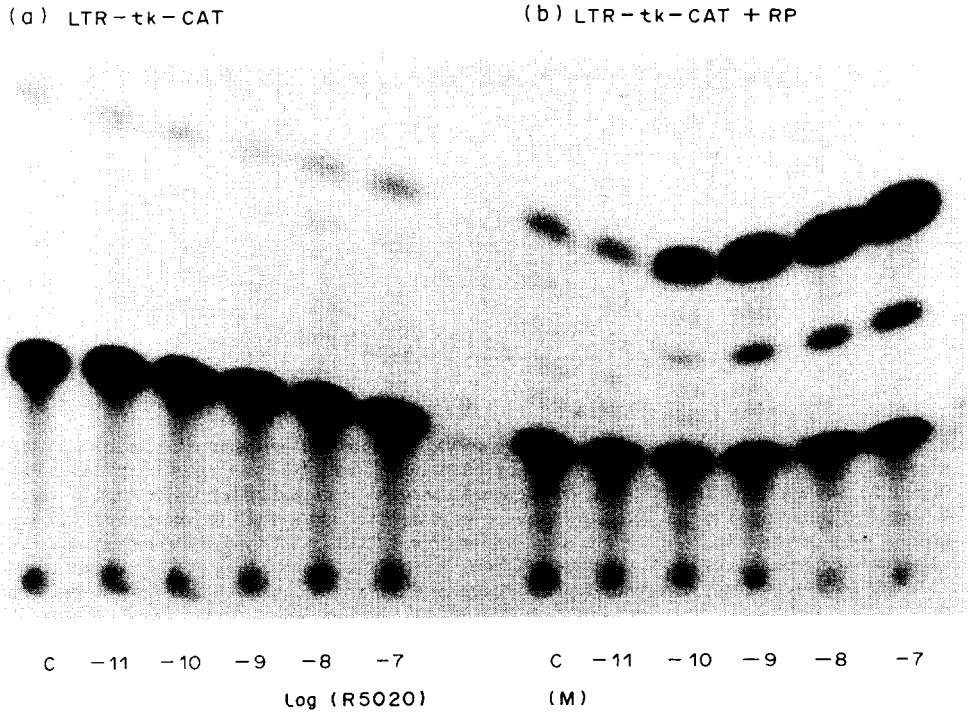

Fig. 7. Effect of transfected human RP on the induction of LTR-tk-CAT in Ishikawa cells deprived of endogenous RP. Following 1 week of steroid-deprivation, Ishikawa cells seeded in 6-well multidishes were transfected with the PR recombinant LTR-tk-CAT (3 μg) alone (a), or with both hPR1 and hPR2 (0.3 μg each). Transiently transfected cells were then incubated for 2 days with the indicated concentrations of R5020. Expression of CAT in cell extracts was then measured as described in experimental procedure.

cell growth. First, estrogen responsiveness of the cath-D gene seems to be easily recovered by transfection of the RE gene. By contrast, the control of cell growth by estrogen or antiestrogen appears to be lost, thus suggesting that other mitogens probably stimulate cell growth and that the cells have lost their ability to be stimulated for growth by the estrogen–RE complex and to be inhibited by the antiestrogen–RE complex. MDA-MB 231 cells could be controlled by autocrine or intracrine growth factors.

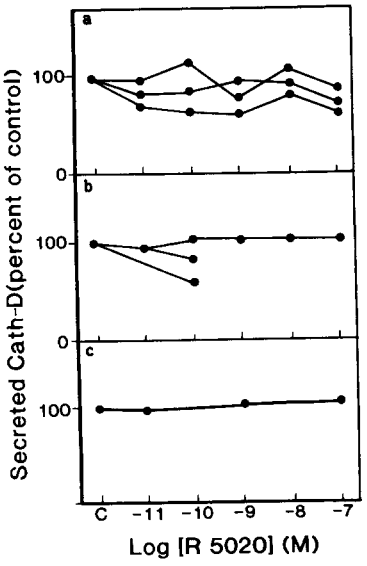

Fig. 8. Dose response effect of R5020 on RP transfected Ishikawa clones. Three Ishikawa cell clones transfected with the RP form A (a), form B (b) and one Ishikawa clone transfected with both RP forms were studied. Cells were withdrawn as described in Fig. 7, then treated for 3 days with vehicle (ethanol 1‰) or with the indicated R5020 concentrations. Secreted cath-D concentrations were then determined by immunoradiometric assay.

However, cath-D gene regulation does not seem to be altered compared to other RE-positive breast cancer cell lines (such as MCF7 cells) since it is stimulated by E2, but not by antiestrogens. This contrasts with antiestrogen resistant variants (R27, RTX6) which are RE-positive, and in which the cath-D gene can be induced by tamoxifen [21].

Contrary to the other cell lines, the cath-D gene remained unresponsive to both estrogens and progestins in Ishikawa cells. As in this cell line endogenous RP [3, 22] and alkaline phosphatase [23] are responsive to estrogens, we suggest that the apparent unresponsiveness of the cath-D gene is due to a general loss of responsiveness to progesterone in Ishikawa cells. We checked the effects of transfection of the normal human RP gene into these cells and observed that the cath-D gene remains

unresponsive to progestins. These results suggest that cooperative transcription factor(s) specific for the progesterone responsive genes is (are) lacking, or alternatively that the hormone responsive element of the cath-D gene is modified. If this can be demonstrated, regulation of the cath-D gene would be due to estrogen in mammary cells and uterine cervix and to progesterone in both normal and cancerous endometrium. However, regulation of cath-D in another endometrial progesterone responsive cancer cell lines and in normal mammary cells transfected with the expression vector for the RE gene should be studied before concluding that the transformation process does not modify the specificity of its hormonal regulation.

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