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The Rat Endometrial Adenocarcinoma Cell Line RUCA-I: a Novel Hormone-responsive *In Vivo/In Vitro* Tumor Model

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The recently established, estrogen receptor positive rat endometrial adenocarcinoma cell line RUCA-I was tested for estrogen responsiveness *in vivo* and *in vitro*. *In vivo*, 10^6 RUCA-I cells were injected subcutaneously into intact, ovariectomized, or ovariectomized, estradiol-substituted syngenic DA/Han rats. All animals developed well differentiated endometrial adenocarcinoma, that had metastasized to peripheral lymph nodes and into the lung. Ovariectomy reduced tumor and lymph node weight, as well as number of lung metastases significantly compared to controls. In another series of experiments, treatment with the pure anti-estrogen ZK 119010 basically gave the same results as seen in ovariectomized animals, whereas tamoxifen treatment had no effect on metastasis of RUCA-I cells. These findings clearly demonstrate the estrogen dependency of growth and metastasis of RUCA-I cells *in vivo*. *In vitro*, we assessed the estrogenic and anti-estrogenic potency of various anti-estrogens, thereby investigating their effects on the expression of components of the complement C3 complex as an estradiol-induced protein and on the expression of fibronectin as an estrogen-repressed protein. Evaluating the relative anti-estrogenic potency of these anti-estrogens we found that ICI 164384 and ICI 182780 behaved as complete antagonists in vitro. Tamoxifen, like estradiol, stimulated complement C3 production and repressed fibronectin expression and has to be regarded as an agonist in this particular in vitro system. ZK 119010 if given alone had no significant influence on the biosynthesis of complement C3 and of fibronectin if compared to the unstimulated control. In addition, another estrogen dependent parameter was identified. Estrogen and anti-estrogen treatment affected glycosylation of complement C3 components. After estradiol treatment predominantly the higher glycosylated epitope of complement C3 became detectable, which could be transformed into the low molecular weight epitope by treatment with hyaluronidase. Finally, we compared the anti-proliferative effects of ICI 164384 and of tamoxifen *in vitro*. Both anti-estrogens slightly inhibited the growth of RUCA-I rat endometrial adenocarcinoma cells. In conclusion, RUCA-I cells represent a powerful, endometrial derived experimental model to test the agonistic and antagonistic properties of anti-estrogens on growth and metastasis *in vivo* and on gene expression *in vitro*. The effects of the tested anti-estrogens on gene expression of RUCA-I cells were found to be useful in predicting their effectiveness on tumor growth *in vivo*. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

In vivo the key role of estrogens in the regulation of proliferation and differentiation of normal and malignant endometrial tissue of humans [1] and rodents [2] is established for many years. Despite this clearcut estrogen dependency of growth and differentiation of the normal endometrium and endometrial adenocarcinoma, no effective anti-estrogen therapy has been established so far. Some attempts have been made to treat endometrial cancer with the anti-estrogen tamoxifen [3], the standard treatment for hormone dependent metastatic breast cancer [4] or by toremifene [5]. However, there exists considerable concern that these hormones may exert partial agonistic activity in the uterus and therefore may cause an increased risk for

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endometrial cancer as seen in breast cancer patients after long term treatment with tamoxifen [4, 6]. More beneficial in terms of cancer treatment may be so called "pure" anti-estrogens, which exert no apparent estrogenic effect, e.g. the anti-estrogens ICI 164384 [7], ICI 182780 [8], ZM 189154 [9] and ZK 119010 [10] which completely block estrogenic effects in the normal uterus.

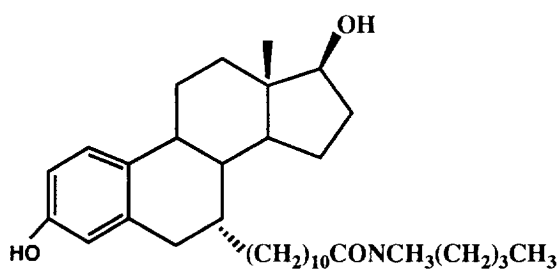
However, investigations on therapeutic efficacy and on molecular aspects of estrogen and anti-estrogen function in normal and malignant endometrial tissues are often limited because of the lack of suitable cell culture models. We recently published the establishment and characterization of (a) the transplantable, estrogen responsive, metastasizing EnDA rat endometrial adenocarcinoma [11] and (b) the estrogen receptor (ER) positive rat endometrial adenocarcinoma cell line RUCA-I [12], which has been derived from an EnDA tumor.

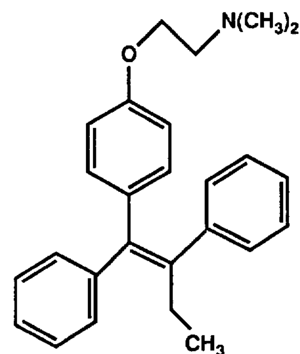
When the EnDA-tumor was subjected to therapy with anti-estrogens, it was immediately apparent that the treatment success was dependent on the kind of anti-estrogen applied. Whereas the almost pure anti-estrogen ZK 119010 reduced tumor weight, the weight of ipsilateral lymph nodes and the number of lung metastases significantly, tamoxifen treatment was ineffective in the reduction of the weight of the primary tumor, enhanced the weight of ipsilateral lymph nodes and increased the number of lung metastastases if compared to untreated controls. These experiments clearly documented the hormone responsiveness of this tumor model [13].

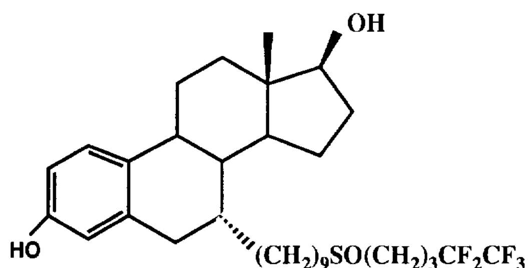
RUCA-I endometrial adenocarcinoma cells, if cultured on a reconstituted basement membrane (Matrigel™, Harbor-matrix™) in the presence of a serum-free defined medium (SFDM), responded to estrogen treatment by an increased growth rate, by the increased production of components of the complement C3 complex [14] and by the repression of fibronectin biosynthesis [15].

To prove finally that RUCA-I cells represent a suitable model system to study functions and therapeutic efficacy of anti-estrogens in endometrial cancer it is the need to fulfil the following criteria: (1) *in vivo*, estrogen-sensitive tumors should be formed if cells are inoculated into syngenic animals; (2) there should be a differential response to treatment with pure anti-estrogens and those anti-estrogens with mixed agonistic and antagonistic properties respectively, both *in vivo* and *in vitro*.

With the study presented here, we investigated the *in vivo* properties of RUCA-I cells by inoculating them subcutaneously into syngenic DA/Han rats and treating them by ovariectomy and by anti-estrogens. We thereby







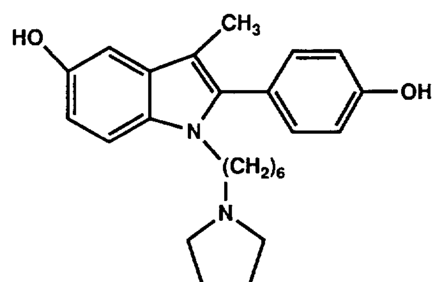


Fig. 1. Antiestrogens: shown are the molecular structures of the antiestrogens ICI 164384, ICI 182780, Tamoxifen and ZK 119010, which were used in this study.

used ZK 119010 as a representative of an almost pure anti-estrogen and tamoxifen as a compound shown to exert mixed antagonistic and agonistic functions. *In vitro*, we extended our studies and investigated the effects of tamoxifen, ZK 119010 and additionally of ICI 164384 and of ICI 182780 on gene expression in RUCA-I rat endometrial adenocarcinoma cells. In comparison to an untreated control and to RUCA-I cells treated by estrogen, we assessed the relative potency of the these anti-estrogens in the expression of complement C3 components and of fibronectin. Furthermore, we detected effects of estradiol and anti-estrogens on the glycosylation of complement C3 components.

MATERIAL AND METHODS

Hormones

17β-Estradiol was from Sigma (Deissenhofen, Germany), ICI 164384 was provided by Dr A. E. Wakeling (Zeneca, Macclesfield, U.K.), and ZK 119010 and ICI 182780 were synthesized at Schering AG (Berlin, Germany).

Cell culture

From RUCA-I cells large quantities of cells of passages 10, 30 and 50 were grown, frozen and subsequently stored in liquid nitrogen. This enabled us to use the same stock of cells for all experiments. Prior to experimental use, RUCA-I cells were precultured for one passage in DMEM/F12 medium without Phenol Red containing 10% fetal calf serum and for two passages in the above medium containing 5% dextran-coated charcoal treated fetal calf serum (DCC-FCS). Thereafter cells were harvested and 250,000–300,000 cells were seeded on top of 300 μl of ECM-substrate (Harbour matrix: Cell Systems, Germany) per well of a 24-well plate in the presence of 2 ml serum-free defined medium (SFDM). The SFDM was composed of DMEM/F12 and contained additionally 2 μg/ml insulin, 4 mM glutamine, 40 μg/ml transferrin, 10^{-8} M hydrocortisone, 2×10^{-8} M sodium selenite and 1 μg/mi putrescine. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C and medium was changed twice a week except for Matrigel cultures, which were fed daily.

Use of RUCA-I cells in in vivo *experiments*

RUCA-I endometrial adenocarcinoma cells were precultured using standard cell culture conditions (plastic and medium containing 10% charcoal stripped fetal calf serum) and harvested by trypsination. Seven to eight-week-old female DA/Han rats were randomized in groups of eight to 10 animals and 10^6 RUCA-1 cells were subcutaneously injected into the right flank.

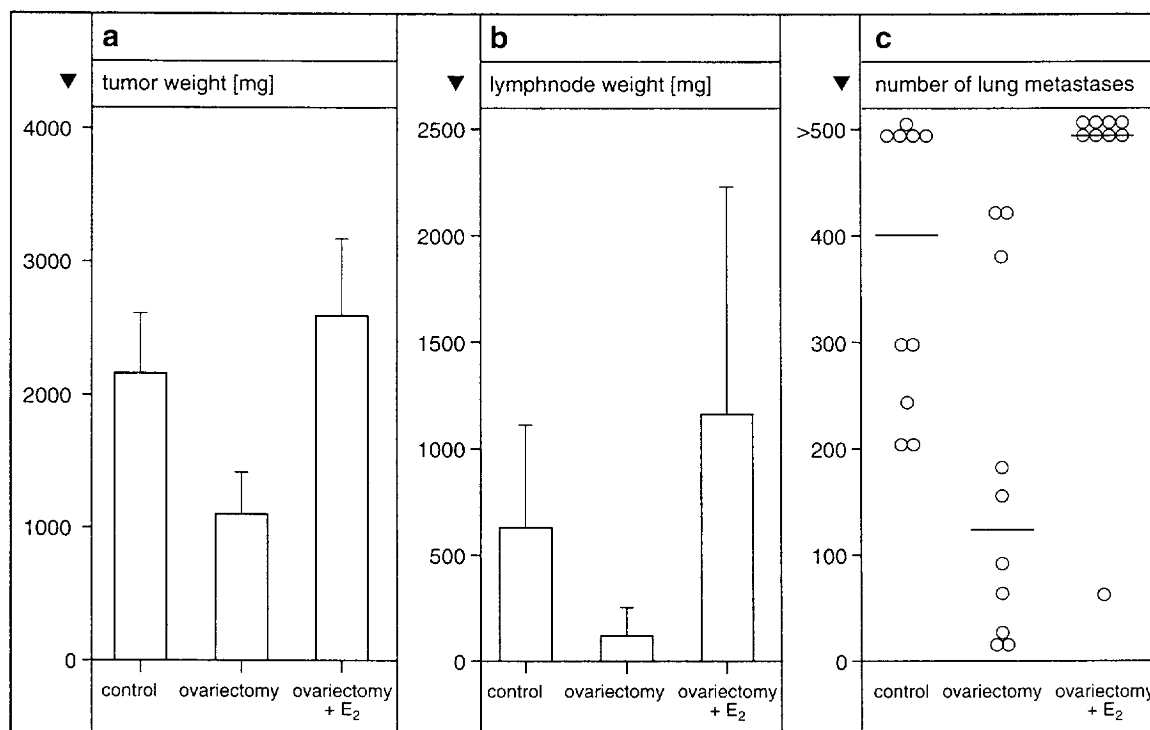


Fig. 2. Effect of hormone deprivation on primary and metastatic tumor growth of RUCA-I cells. 10^6 RUCA-I cells were s.c. injected into the right flank of intact syngenic female DA/Han rats, ovariectomized DA/Han rats and ovariectomized DA/Han rats substituted with estradiol-undecylate. The effect of these treatments on tumor weight (a) mean + SD, weight of ipsilateral lymph nodes (b) mean + SD and on the number of lung metastases (c) is given. In Fig. 2c each circle represents the number of superficial visible lung metastases of a single rat. The horizontal bars indicate the median number of lung metastases.

For hormonal deprivation, rats were ovariectomized under ether anaesthesia 48 h prior to tumor implantation. Estradiol substitution of ovariectomized rats was performed by a single-depot injection of estradiol undecylate (Progynon Depot 100, Schering) diluted in oil (20% benzyl benzoate in castor oil) 24 h before tumor implantation. The amount of estradiol undecylate injected was 50 μg/rat and the injection volume was 100 μl. This estradiol dose was sufficient to stimulate EnDA-tumor growth in ovariectomized DA/Han rats to the level of intact animals for at least 6 weeks [13].

For endocrine treatment, drugs were dissolved in oil (20% benzyl benzoate in castor oil) and applied by s.c. injection. Treatment was performed 6 days a week for 5 weeks starting 1 day after tumor cell injection. The chosen dosages were 20 mg/kg tamoxifen and 5 mg/kg ZK 119010. After 5 weeks the animals were killed, the tumors, lymph nodes and uteri were excised and the wet weights were determined.

For quantitative evaluation of the number of lung metastases, lungs of all animals were removed and fixed in Bouin's solution. After fixation for 24 h, metastases were outlined as opalescent white areas. The number of visible superficial lung metastases was counted under the dissecting microscope.

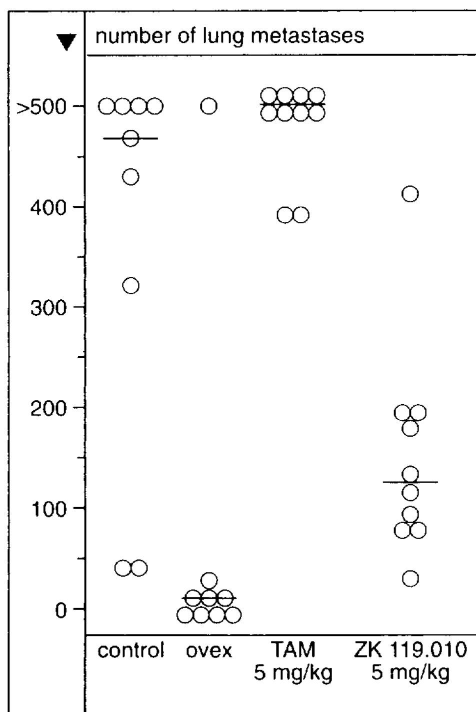


Fig. 3. Effect of antiestrogen treatment on the number of lung metastases after s.c. injection of RUCA-I cells. The effect of tamoxifen (20 mg/kg) and of ZK 119010 (5 mg/kg) on the number of lung metastases after s.c. injection of 10^6 RUCA-I cells into the right flank of intact female DA/Han rats was assessed. The effectiveness of treatment was compared to untreated control animals and to the tumor growth on ovariectomized animals. Each circle represents the number of superficial visible lung metastases of a single rat. Horizontal bars indicate the median number of lung metastases.

Hormonal treatment of RUCA-I cells in cell cultures

For hormonal treatment we used estradiol (10^{-7} mol/1) as agonistic ligand; as anti-estrogens we used ICI 164384 [16], ICI 182780 [17], ZK 119010 [10] at 5×10^{-7} mol/l and tamoxifen at 10^{-6} mol/l [18]. This means hormonal components were used in approximately equipotent concentrations because of their relative binding affinities to the ER. Prior to hormonal treatment, cells were seeded on ECM in the above numbers and precultured in SFDM on the substrate for 24–48 h. Thereafter cells were incubated for 48 h with estradiol or ICI 164384. Control cultures received ethanol, which was used as a vehicle. Medium was changed daily and hormonal treatment was repeated. In some experiments additional controls were included by culturing RUCA-I cells in 24-well plates on plastic in the presence of DMEM/F12 medium containing 5% DCC-FCS or SFDM and the above concentrations of hormone or anti-hormone.

Metabolic labelling of secretory proteins

After 48 h of hormonal stimulation, synthesized proteins were labelled metabolically with ^{35}S-methionine. During labelling, cells were cultured for another 16 h in the above media and under the hormonal conditions described above, including the following modifications: the methionine content of the medium was reduced by 90%; instead the medium was substituted with 200 μCi/ml ^{35}S-methionine. The volume of the cell culture medium per well was reduced to 150 μl. At the end of the incubation period cell culture supernatants containing the *de novo* synthesized metabolically labelled secretory proteins were aliquoted in a 15 μl aliquot and an aliquot, containing the remainder. From the 15 μl aliquot, the incorporation rate of the radioactive amino acid was determined, and the remainder was used for SDS–PAGE electrophoresis. Total synthesis of secretory proteins was determined in triplicate by precipitating proteins out of 3 μl cell culture supernatant with TCA.

In order to determine whether or not investigated proteins represent glycoproteins, cell culture supernatants were digested at room temperature for 12 h with 10 mg/ml or 1 mg/ml of hyaluronidase (Boehringer. Mannheim, Germany) prior to electrophoretic separation.

Electrophoresis

Electrophoresis was performed according to standard protocols using a discontinuous system [19], reducing and non-reducing conditions and either 5%, 7.5% or 10% polyacrylamide gels. Loading of the gel was performed after the amount of acid precipitable protein had been determined. For each experimental condition the same number of counts, meaning the same amount

of protein-bound ^{35}S-methionine was loaded on the gel. After electrophoresis the gels were fixed, incubated in En^{3}Hance (DuPont, Germany), dried and the gel bands were visualized by fluorography. For semiquantitative evaluation, autoradiographs were subjected to densitometry (Hoefer, U.S.A.).

Proliferation

Cell multiplication was measured with the EZ4U test (Biomedica, Vienna, Austria) according to the manufacturer's protocol. This test basically is an advanced *in vivo* MTT-test [20]. We performed this test by determining each individual experimental condition 8-fold in 96-well microtiter plates. For measurements on plastic, 1000 cells were seeded per well of the multiwell plate in the presence of 10% fetal calf serum or SFDM. The bottom of another subset of wells was covered with 30 μl of reconstituted basement membrane. In these experiments 2000 cells were seeded on top of the ECM substrate. After 1-, 3- and 4 days of culture, substrates for the EZ4U-test were added and the cell proliferation was assessed by reading the extinction of the colour dye.

RESULTS

Effect of hormone deprivation on primary and metastatic tumor growth of RUCA-I cells in vivo

As reported elsewhere [12] RUCA-I cells give rise to endometrial adenocarcinoma if subcutaneously inoculated into syngeneic DA/Han rats; however, the hormone-responsiveness of these tumors originating from RUCA-I cells has never been checked. In a first series of experiments we therefore tested if primary and metastatic tumor growth of RUCA-I cells respond to hormone deprivation. Ovariectomy significantly reduced tumor weight (Fig. 2a), weight of ipsilateral lymph nodes (Fig. 2b) and the number of lung

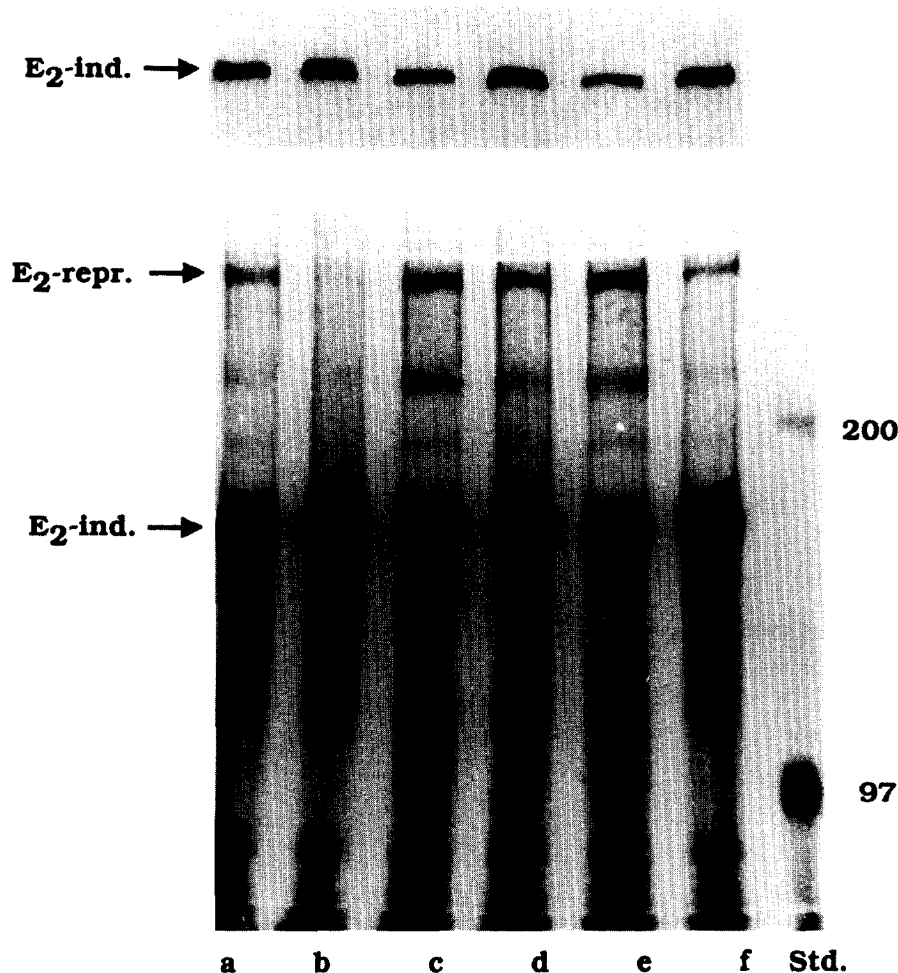


Fig. 4. Induction and repression of secreted proteins by estrogens and antiestrogens. Proteins secreted by RUCA-I cells were metabolically labelled with ^{35}S-methionine and separated on a 5% Polyacrylamide gel using non-reducing conditions. Shown are secreted proteins from untreated RUCA-I cells (a), or from RUCA-I cells treated with the following hormones and antihormones: estradiol 10^{-7} mol/l (b), ICI 164384 5×10^{-7} mol/l (c), ZK 119010 5×10^{-7} mol/l (d), ICI 182780 5×10^{-7} mol/l (e) and tamoxifen 10^{-6}mol/l (f). Labelled are the estradiol-induced (E_2-ind.) complement C3 protein and the estradiol-repressed (E_2-repr.) glycoprotein fibronectin. The upper part of the figure represents a shorter exposure of the E_2-ind. complement C3.

metastases (Fig. 2c). All effects of ovariectomy could be counteracted by substitution of animals with estradiol-undecylate (Fig. 2a-c). These results provide clearcut evidence for the estrogen dependency of primary and metastatic tumor growth of RUCA-I cells *in vivo*.

Effects of anti-estrogen treatment on metastatic tumor growth of RUCA-I cells.

To test whether endocrine treatment is capable of reducing metastatic tumor growth of RUCA-I cells we performed treatment of animals by anti-estrogens. As model compounds we used ZK 119010 as an almost complete antagonist and tamoxifen as a representative for a drug with mixed agonistic and antagonistic functions and compared their effects to untreated control animals and hormone deprivation by ovariectomy. ZK 119010 significantly reduced numbers of lung metastases of RUCA-I cells (Fig. 3); however, the reduction of numbers of lung metastases was slightly less than detectable following ovariectomy. In contrast, tamoxifen was ineffective in reducing metastasis of RUCA-I cells to the lung (Fig. 3).

These *in vivo* experiments clearly indicated that primary and metastatic tumor growth of RUCA-I cells is estrogen-dependent and additionally, differentially responds to pure or partial anti-estrogens. We further addressed the question of whether or not this distinct behavior of RUCA-I cells in response to endocrine therapy could be mimicked *in vitro*. To answer this question in a slightly modified approach we investigated the effects of the anti-estrogens ICI 164384, ICI 182780, ZK 119010, and tamoxifen on their estrogenic and anti-estrogenic effects on RUCA-I cells *in vitro*.

Anti-estrogens and expression of complement C3

If the ER positive rat endometrial adenocarcinoma cell line RUCA-I is cultured on a reconstituted basement membrane, cells respond to treatment with estradiol or the anti-estrogen ICI 164384. In this experimental *in vivo* model, complement C3 has been shown to be an estradiol inducible protein [14], whereas fibronectin expression was found to be estrogen-repressed [15].

To assess the relative antagonistic or agonistic properties of the anti-estrogens ICI 164384, ICI 182780, ZK 119010 and tamoxifen experimentally, RUCA-I cells were treated with these anti-hormones only without a pretreatment of cells with estradiol or simultaneous treatment with estradiol (competitive analysis). After hormonal treatment cell culture supernatants were collected, protein bound ^{35}S-methionine was estimated and equal numbers of radioactive counts of each experimental condition were subjected to SDS–PAGE. Both, reducing and non-reducing conditions were used in order to analyse complement C3 as either a single molecule (non-reduced) or individual components (reduced).

Treatment of RUCA-I cells by estradiol leads to a statistically significant increase in complement C3 production if compared to an untreated control. This

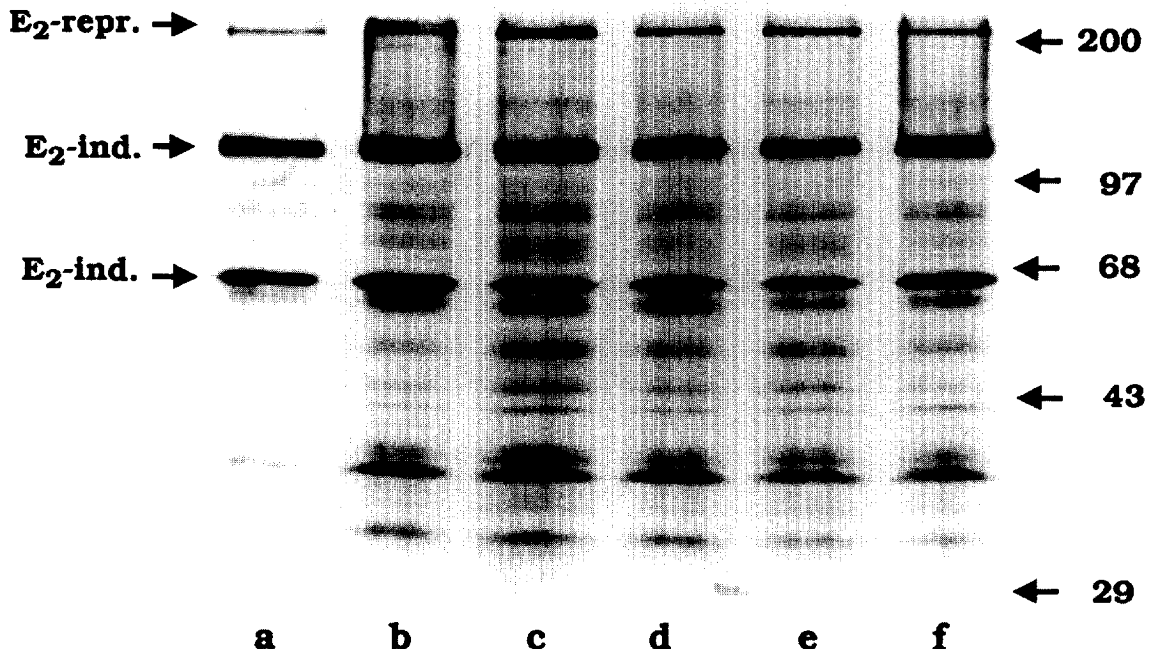


Fig. 5. Induction and repression of secreted proteins by estrogens and antiestrogens. Proteins secreted by RUCA-I cells were metabolically labelled with ^{35}S-methionine and separated on a 7.5% polyacrylamide gel using reducing conditions. Shown are secreted proteins from untreated RUCA-I cells (a), or from RUCA-I cells treated with the following hormones and antihormones: estradiol 10^{-7} mol/l (b), ICI 164384 5×10^{-7} mol/l (c), ZK 119010 5×10^{-7} mol/l (d), ICI 182780 5×10^{-7} mol/l (e) and tamoxifen 10^{-6} (f). Labelled are the estradiol-induced (E_2-ind.) complement C3 protein and the estradiol-repressed (E_2-repr.) glycoprotein fibronectin.

finding is clearly detectable for both, analysis under non-reducing (Fig. 4b; Fig. 6b) and reducing (Fig. 6b: Fig. 6a) conditions for polyacrylamide gel electrophoresis, as demonstrated for both the 115 kD subunit (HMW) and 60 kD (LMW) of complement C3 (Fig. 5b; Fig. 6a), detectable in an analysis using reducing conditions. The formation of this protein was most efficiently repressed after treatment of cells with ICI 164384 (Fig. 4c, Fig. 5c, Fig. 6a,b). In an analysis using non-reducing conditions we additionally compared the efficiency of ICI 164384 and ICI 182780 on the repression of complement C3 expression (Fig. 4c,e; Fig. 6b). The two components were almost equally potent in repressing complement C3 protein. The two other anti-estrogens ZK 119010 and tamoxifen increased production of complement C3 production by RUCA-I cells. The effect of ZK 119010 (Fig. 4d; Fig. 5d; Fig. 6a,b) was only marginally above the levels of the untreated control (Fig. 4a, 5a, 6a,b), whereas tamoxifen treatment led to a more pronounced increase in complement C3 production (Fig. 4f; Fig. 5e; Fig. 6a,b), which was statistically significant for the 60 kD subunit (LMW; Fig. 6a) in the analysis under reducing conditions.

Effects of anti-estrogens on fibronectin expression

Treatment of RUCA-I cells with estradiol (Fig. 4b; Fig. 5b; Fig. 7a,b) significantly repressed secretion of fibronectin far below levels observable in untreated controls (Fig. 4a; Fig. 5a; Fig. 7a,b). Since fibronectin is an estradiol repressed protein, its production is most efficiently stimulated by treatment of RUCA-I cells with the pure anti-estrogens ICI 164384 (Fig. 4b; Fig. 5b; Fig. 7a,b) or ICI 182780 (Fig. 4e; Fig. 7b). There was no significant difference detectable in the potency of these two components if their efficiency was compared on a non-reducing gel (Fig. 4c,e; Fig. 7b). The two other anti-estrogens tested, slightly repressed fibronectin production by RUCA-I cells. The effects detectable for ZK 119010 again were rather marginal (Fig. 4d; Fig. 5d; Fig. 7a,b), whereas the effect of tamoxifen at least under non-reducing conditions was found to be statistically significant (Fig. 4f; Fig. 5e, Fig. 7a,b).

Estrogenic effects on glycosylation

Depending on the quality of the resolution of the non-reducing gels we were able to resolve either one (E2-ind.; Fig. 4, 9) or two bands for the non-reduced complement C3 complex (E2-ind.; Fig. 8), particularly after estradiol treatment of RUCA-I cells. Since this effect in the non-reducing approach was not attributable to different subunits, we speculated that it might be caused by differential glycosylation of the molecule. We tested various glycosidases and with the help of hyaluronidase we could partially (Fig. 9e) or completely (Fig. 9d) convert the non-reduced high molecular weight complement C3 complex secreted by RUCA-I

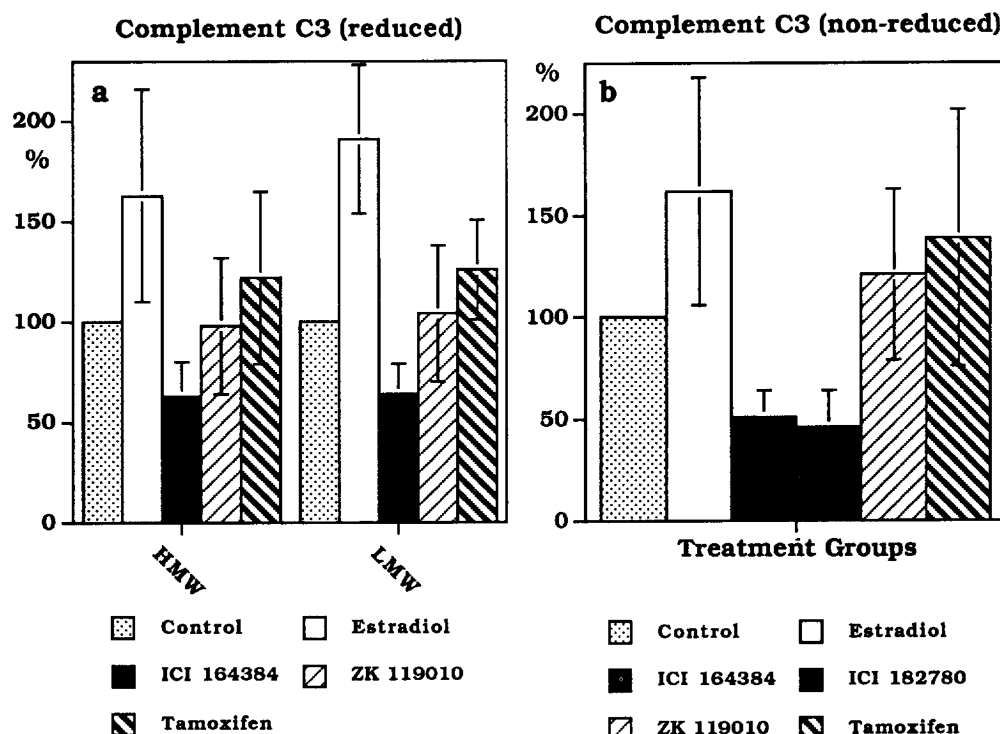


Fig. 6. Quantitative evalulation of the estradiol-induced complement C3 components. For quantitative evaluation eight experiments performed under reducing conditions (a) and five experiments performed under non-reducing conditions (b) were subjected to semiquantitative densitometry. Results obtained for treatment with estradiol and ICI 164384 or ICI 182780, as well as for tamoxifen on the low molecular weight (LMW) chain of complement C3 were statistically significant.

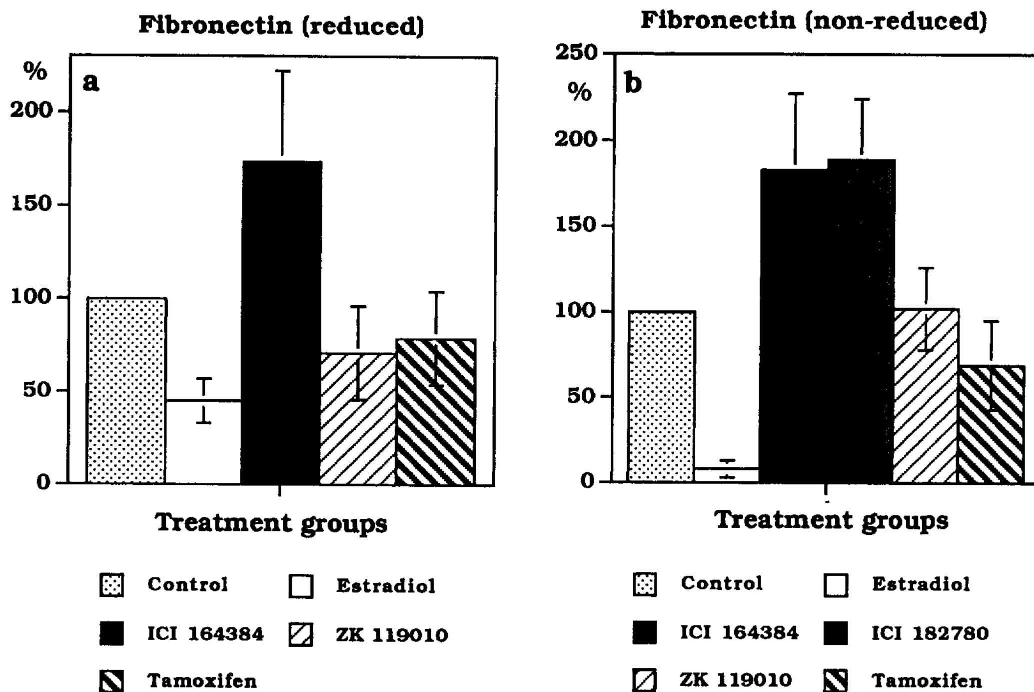


Fig. 7. Quantitative evalulation of the estradiol-repressed glycoprotein fibronectin. For quantitative evaluation eight experiments performed under reducing conditions (a) and five experiments performed under non-reducing conditions (b) were subjected to semiquantitative densitometry. Results obtained for treatment with estradiol and ICI 164384 or ICI 182780, as well as for tamoxifen in the non-reduced approach (b) were statistically significant.

cells following estradiol treatment into a low molecular weight epitope. In gels with a high resolution, as shown in Fig. 8, we also observed a dependency of glycosylation on the relative agonistic or antagonistic capacity of the hormonal derivative used to stimulate RUCA-I cells.

Densitometric analysis of high resolution gels revealed that after estradiol treatment not only an increase in total complement C3 production was observable (Fig. 10), but also an increase in the relative amount of the higher glycosylated epitope (dashed columns), which was the dominating isoform synthesized by estradiol treated cells. In cells treated by the ICI components we never observed any highly glycosylated isoforms (Fig. 8c,e; Fig. 10). Like in cell culture supernatants from untreated controls (Fig. 8a; Fig. 10) in cell culture supernatants from cells treated with either ZK 119010 (Fig. 8d) or tamoxifen (Fig. 8f) the low molecular weight epitope of complement C3 dominated; however, lower amounts of the highly glycosylated isoform always persisted (Fig. 8d,f; Fig. 10). The latter finding again provides some evidence for the mixed agonistic/antagonistic features of these anti-estrogens.

Proliferation

Measurements of proliferation in cultures on a reconstituted basement membrane are hard to perform, since neither can cells be released as single cell suspensions, nor can antibody directed against proliferation antigens be used, since these IgG molecules very tightly bind to the matrix-components. Although not completely satisfactory, the working proliferation test is the EZ4U-test, a modified MTT-test [20]. Using this test we compared cell growth in cultures of RUCA-I cells either grown without a hormonal treatment or grown in the presence of 10^{-8} mol/l estradiol, 5×10^{-8} mol/l ICI 164384 or 10^{-6} mol/l tamoxifen. Estradiol stimulated cell growth (Fig. 11); this effect was statistically significant after 4 days in culture. Treatment of cells with anti-estrogens slightly reduced the growth of RUCA-I cells; however this trend was not found to be statistically significant (Fig. 11).

DISCUSSION

We tested the estrogen and anti-estrogen sensitivity of growth and metastasis of the novel, ER-positive rat endometrial adenocarcinoma cell line RUCA-I *in vivo* and on proliferation and gene expression *in vitro*. In comparative experiments tamoxifen, if given alone, behaved merely as an estrogen, both *in vivo* and *in vitro*. Treatment with ZK 119010 showed almost complete estrogen antagonism *in vivo*; however, *in vitro* results were almost indistinguishable from the untreated controls. Both ICI compounds were only tested *in vitro*, but in this situation behaved as pure anti-estrogens, thereby inducing the increased biosynthesis of fibronectin, an estradiol-repressed protein in RUCA-I cells. From these results we conclude that primary and

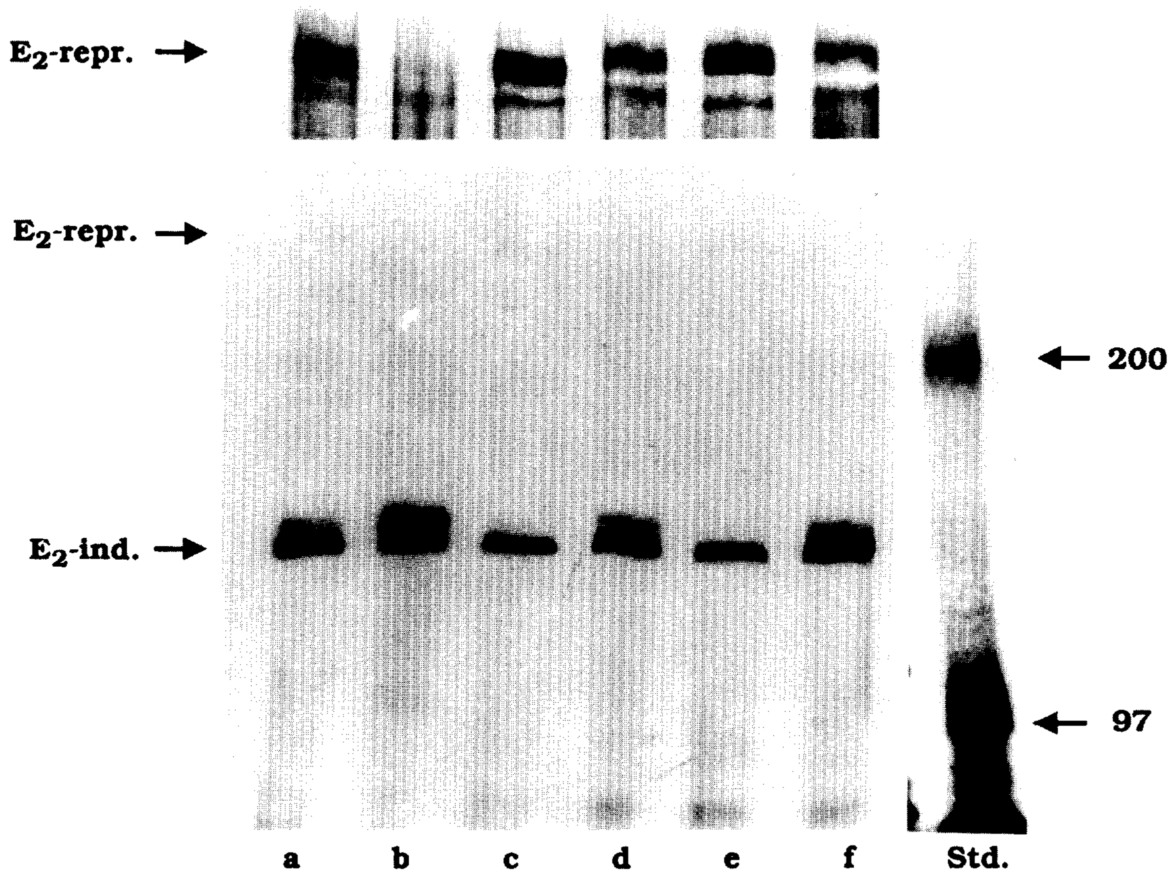


Fig. 8. Different subunits of the estradiol-induced complement C3. Proteins secreted by RUCA-I cells were metabolically labelled with ^{35}S-methionine and separated on a 5% polyacrylamide gel using non-reducing conditions. Shown is a gel with a high resolution of secreted proteins from untreated RUCA-I cells (a), or from RUCA-I cells treated with the following hormones and antihormones: estradiol 10^{-7} mol/l (b), ICI 164384 5×10^{-7} mol/l (c), ZK 119010 5×10^{-7} mol/l (d), ICI 182780 5×10^{-7} mol/l (e) and tamoxifen 10^{-6} mol/l (f). Labelled are the estradiol-induced (E_2-ind.) complement C3 protein and the estradiol-repressed (E_2-repr.) glycoprotein fibronectin. The upper part of the figure represents a longer exposure of the E_2-repr. glycoprotein fibronectin.

metastatic tumor growth of RUCA-I cells *in vivo* and their estrogen and anti-estrogen dependency of gene expression *in vitro,* strictly depend on the treatment protocol and the type of anti-estrogen used. This finding further implies that RUCA-I cells represent a potent and representative *in vivo* and *in vitro* model to test for estrogenic and anti-estrogenic properties of estrogens and anti-estrogens and, furthermore, an *in vitro* model to test natural and synthetic compounds on their estrogenicity in endometrial derived cells.

In vivo, estrogens play a key role in the regulation of proliferation, differentiation and gene expression in the normal endometrium and in endometrial adenocarcinoma of humans [1] and rodents [2]. Therefore, endometrial-derived *in vitro* models that allow estrogenic and anti-estrogenic properties to be assessed have clinical and pharmacological impacts, since up to date no effective treatment of endometrial adenocarcinoma with anti-estrogens, e.g. tamoxifen, the standard treatment for hormone dependent breast cancer [4], has been established. The failure in the establishment of a standard anti-estrogen treatment protocol for the treatment of endometrial adenocarcinoma is due to the considerable concern that these hormones may exert partial agonistic activities in the uterus and give rise to increased risk for endometriosis [21–23] and endometrial adenocarcinoma [24, 25]. A representative *in vitro* model is therefore important for two experimental purposes: (a) evaluation of anti-estrogens which are currently in clinical use; and (b) particularly for the screening of novel anti-estrogens. It would be advantageous to have a simple endometrial-derived *in vitro* model available which is able to classify anti-estrogens into those components that have agonistic, partial agonistic and complete antagonistic features. RUCA-I cells, if used *in vivo* for primary and metastatic tumor growth or, if cultured on a reconstituted basement membrane, represent a combined *in vitro*/*in vivo* model that fulfils these needs. With investigations on the estrogenic control of growth and metastasis *in vivo* and of the expression of components of the complement C3 complex, of fibronectin and of glycosylation of complement C3 *in vitro,* one can easily dissect agonistic and antagonistic properties of anti-estrogens.

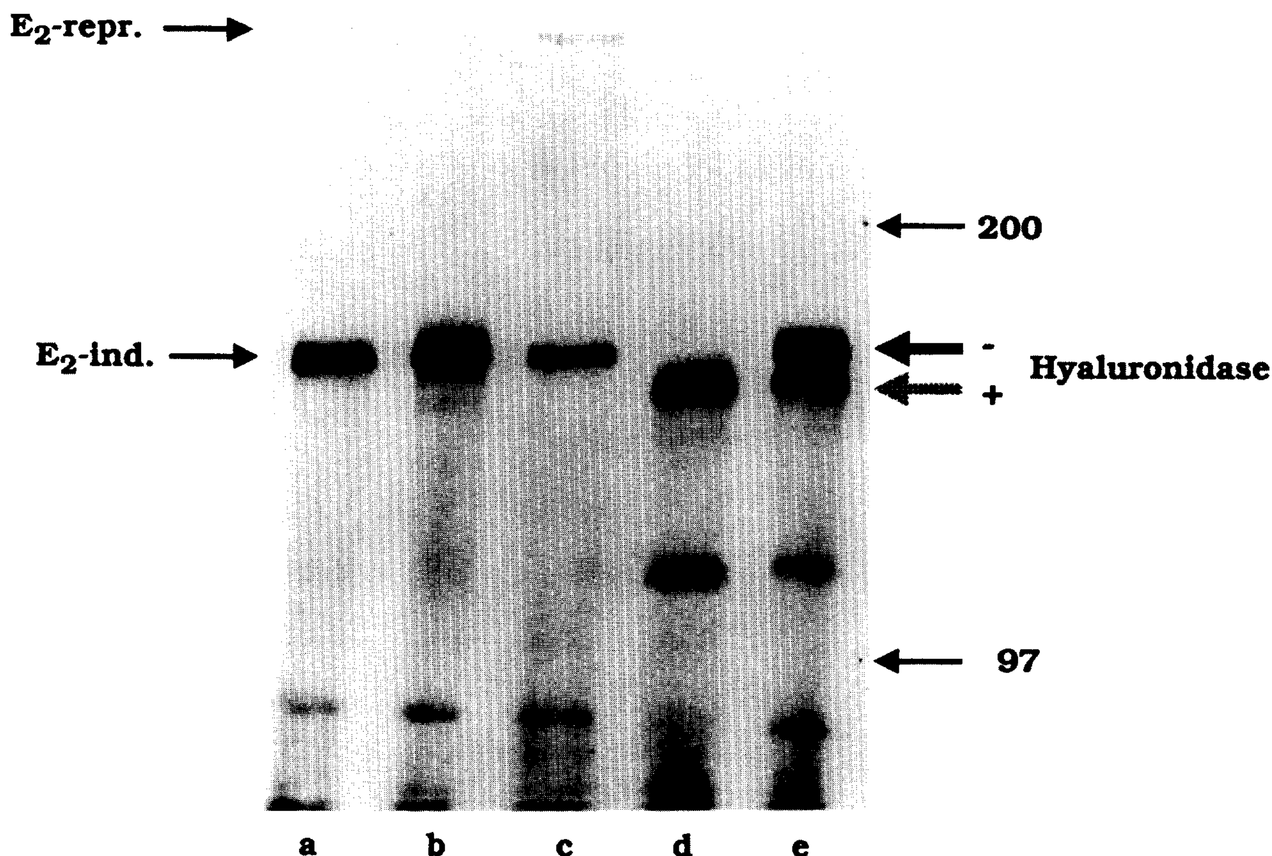


Fig. 9. Hyalurinodase treatment of cell culture supernatants. Proteins secreted by RUCA-I cells were metabolically labelled with ^{35}S-methionine and separated on a 5% polyacrylamide gel using non-reducing conditions. Shown are secreted proteins from untreated RUCA-I cells (a), or from RUCA-I cells treated with the following hormones and antihormones: estradiol 10^{-7} mol/l (b), ICI 164384 5×10^{-7} mol/l (c). This figure also shows hyaluronidase digestion at concentration of 10 mg/ml (d) or 1 mg/ml (e) of cell culture supernatants, from estradiol-treated RUCA-I cells.

In vitro, if RUCA-I cells and the identified estrogen dependent processes are used to test for estrogenic versus anti-estrogenic effects, methodologically, we clearly prefer protein gel electrophoresis under non-reducing conditions over protein gel electrophoresis under reducing conditions. There are several reasons for this:

1. Differences in glycosylation so far only have been resolved under non-reducing conditions.
2. The inductory effects on the formation of complement C3 components and on fibronectin are statistically more pronounced in the non-reduced analysis.
3. The estrogenic regulation of the analyzed proteins appears clearer in non-reduced gels than in reduced gels.

Additionally, our observations of glycosylation of proteins, particularly by sulfated sugar moieties, being an estrogen-dependent process provides us with another experimental parameter and agrees with earlier reports where sulfate uptake into cultured endometrial cells has been described as a progesterone-stimulated process in estradiol primed glandular epithelial cells of the endometrium [26]

Another important point that has to be discussed is the correspondence of results on gene expression to the situation observed *in vivo*. So far we do not know if tumors derived from RUCA-I cells express complement C3 *in vivo*, because until now we were not able to label tumor pieces sufficiently to have a high enough metabolic labelling in the collected tissue culture supernatants. However, in the immature rat uterus complement C3 expression is induced by estradiol [27] and a variety of anti-estrogens including tamoxifen [28]. In contrast, estradiol and anti-estrogen-induced synthesis of complement C3 is blocked by the pure anti-estrogen ICI 164384 [7]. This very closely matches the situation observed *in vitro* where tamoxifen, like estradiol, stimulated an increased complement C3 biosynthesis, whereas ICI 164384 inhibited even basic, non-stimulated production of complement C3. The third anti-estrogen ZK 119010 had no significant influence on complement C3 expression. Comparative

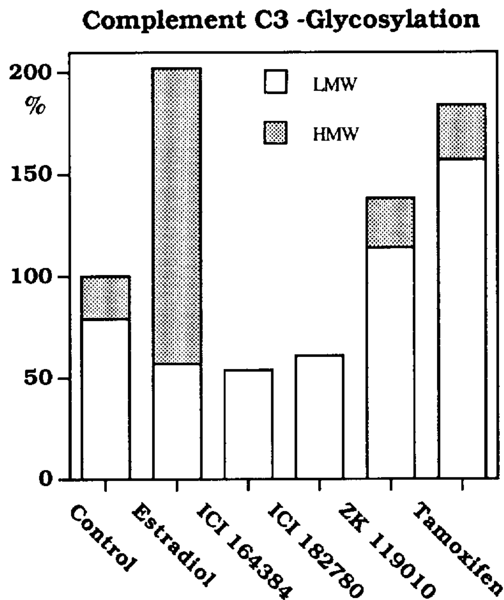


Fig. 10. Glycosylation of complement C3—quantitative aspects. Highly resolved non-reduced cell culture supernatants were subjected to semiquantitative densitometry. The total length of the columns represents the total amount of secreted complement C3. Dashed parts of the columns represent the relative amount of high glycosylated complement C3 (HMW), whereas open parts of the columns represent the low molecular weight complement C3 (LMW).

data on the regulation of fibronectin expression or glycosylation of complement C3 by estrogens *in vivo* are not available.

In a mechanistic view on our results, data obtained by ZK 119010 are difficult to explain. Unlike tamoxifen, ZK 119010 antagonizes the function of estradiol *in vivo*. However, *in vitro* if given alone, it behaves completely differently from the other two anti-estrogens investigated. It neither stimulates gene expression in an estrogenic fashion, nor antagonizes basal expression of estrogen-dependent proteins in the untreated controls, like the ICIs do. Mechanistic studies on the activation of the ER if liganded with ZK 119010 are not available. However, our own observations suggest that ZK 119010 if given alone has merely no intrinsic activity, as would be the explanation for the observations made *in vitro*. However, from our findings *in vivo* and previous findings with MCF-7 breast cancer cells *in vitro* [29], we conclude that ZK 119010 effectively antagonizes the functions of estradiol.

From data in the literature it is known that tamoxifen promotes the DNA binding of the ER [30], and additionally acts as a partial agonist and it has been proposed that its antagonistic properties are derived from an inhibition of the transcription activation function-2 (TAF-2) whereas its agonism derives as a consequence of its TAF-1 activity [31]. These reported activities are sufficient to explain the observed agonistic functions of tamoxifen on RUCA-I cells *in vivo* and *in vitro*.

The molecular mechanism of action of ICI 164384 and of ICI 182780 is still controversially debated in the literature. However, the ICI compounds most likely inhibit the DNA-binding of the ER in many experimental systems [32, 33] by inhibition of the dimerization of the receptor [34]. Additional evidence for this assumption comes from experiments where it has been proven that the ICI compounds are capable of blocking ligand independent activation of the ER [35, 36]. These findings additionally provide a potential explanation for our observation that ICI anti-estrogens can block the unstimulated, estradiol-independent expression of complement C3.

Most difficult to explain are the observed differences of the effects of pure anti-estrogens and of tamoxifen (mixed agonistic/antagonistic) on the growth of RUCA-I cell *in vivo* and *in vitro*. *In vivo* tamoxifen slightly promoted metastasis of RUCA-I cells, although statistically not significant. At least no growth inhibitory effect of tamoxifen was observable. In contrast, the effects of tamoxifen and of ICI 164384 on the growth of RUCA-I cells on matrigel in SFDM could not be discriminated. Whereas estradiol in this model stimulated cell growth significantly, only marginal inhibitory effects were observed following anti-estrogen treatment. This finding neither corresponds to the situation observed *in vivo*, where tamoxifen like estradiol promoted metastasis of RUCA-I cells and additionally

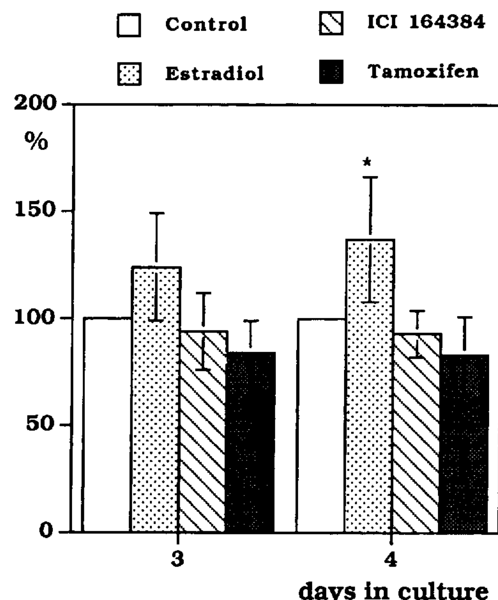


Fig. 11. Hormonal control of growth of RUCA-I cells. Cell growth of RUCA-I cells cultured on a reconstituted basement membrane in the presence of SFDM was measured by the EZ4U-test, a modified MTT-test. Values for the light absorbance of untreated controls after 3 or 4 days in culture were set to 100% and values for estradiol (10^{-7} mol/l), ICI 164384 (5×10^{-7} mol/l) and tamoxifen (10^{-6} mol/l) treated cells were calculated accordingly. Stimulation of cell growth by estradiol after 4 days of treatment was found to be statistically significant.

of the EnDA endometrial adenocarcinoma [13], nor to the previously reported effects of tamoxifen on the inhibition of proliferation of RUCA-I cells if cultured on plastic in the presence of a charcoal stripped fetal calf serum [12]. The growth inhibitory effect of tamoxifen on RUCA-I cells cultured on plastic in the presence of serum is most likely not attributable to an antagonism of the function of the ER. We rather speculate that tamoxifen may negatively regulate growth by interference with the so-called serum responsive element(s) [37].

In conclusion, RUCA-I cells if cultured on a reconstituted basement membrane in the presence of a serum-free defined medium represent a powerful model to screen for estrogenic, anti-estrogenic or mixed estrogenic/anti-estrogenic functions of estrogens and potential anti-estrogens in an endometrial-derived *in vitro* model. In addition, the strength of the model originates from its applicability *in vivo*. Any effect of any tested estrogenic/anti-estrogenic compound can immediately be verified in the *in vivo* situation using appropriate treatment protocols. Further, we propose that RUCA-I cells represent a powerful screening tool for the classification of potential novel anti-estrogens into their estrogenic versus anti-estrogenic activities.

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