Original article

Induction of TGF-beta by an antiprogestin in the human breast cancer cell line T-47D

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Summary

Background: Antiprogestins appear to be a new approach for the endocrine therapy of breast cancer. Most breast cancer cells are growth inhibited by TGF-beta. Any change of tumourcellular TGF-beta secretion could have some impact on tumourcellular growth. We addressed our question to whether the antiprogestin onapristone can induce TGF-beta secretion in breast cancer cells in vitro and whether a possible induction correlates with the antiproliferative effect and the receptor status of the cells.

Materials and methods: We examined the ER and PR positive breast cancer cell lines MCF7 and T-47D and an ER and PR negative variant T-47D/x. Hormone receptor levels were determined by EIA, total (LTGF-beta + active TGF-beta) and active TGF-beta by a radioreceptor assay. All cell biological and antiproliferative effects were measured during basal, not estrogen-stimulated growth.

Results: To our knowledge, we are the first to describe, that the TGF-beta secretion of tumor cells can be increased by an antiprogestin (total: 4.8-fold, active 2.9-fold). A stimulation was found only in the markedly PR positive T-47D cells, in which onapristone proved to have strong antiproliferative potency. In the MCF7 and T-47D/x cells onapristone showed no induction of TGF-beta. Moreover, those cells were not growth inhibited. Whereas onapristone did not influence the ER-content, it dramatically downregulated the PR-content of the T-47D and MCF7 cells (93% and 65%, respectively).

Conclusions: These observations make it likely, that the antiproliferative potency of the antiprogestin onapristone is at least partly due to the ability of onapristone, to stimulate the strong growth inhibitor TGF-beta. In contrast to the antiprogestin RU 486, onapristone showed no estrogenic activity (stimulation of growth and PR), which could be a decisive advantage in the therapy of breast cancer, taking into account, that many breast carcinomas grow estrogen dependent.

Key words: antiprogestins, breast cancer cells, estrogen receptors, onapristone, progesterone receptors, TGF-beta

Background

The endocrine therapy of breast carcinoma with antiestrogens is now established for years. Progesterone antagonists, too, show potent antitumor effects both in vitro and in experimental breast carcinoma of the mouse and rat [1-4]. Progesterone antagonists could enrich the therapeutic facilities of endocrine therapy [5, 6]. Mifepristone (RU 486), the first progesterone antagonist, was developed in the laboratories of Roussel-Uclaf [7], onapristone by Schering AG [8]. Onapristone compared to mifepristone has much less glucocorticoid effects [9], which is acknowledged as a decisive therapeutic advantage. The mechanism of the growth inhibitory effect of the antiprogestins is not clear in all details. We learnt that changed expression of growth factors, among them TGF-β, is crucial for carcinogenesis and that several growth factors can be regulated by different hormones [10, 11]. Most breast cancer cells are growth inhibited by TGF-beta [12, 13]. For that reason any change of tumourcellular TGF-beta secretion could have some impact on tumourcellular growth. Until now in man three different types of TGF-beta are known (TGF-beta-1,2,3), all are potent inhibitors of tumors of epithelial origin via an auto- and paracrine mechanism [14, 15]. Most cells secrete TGF-beta to the largest part in a latent biologic inactive form (LTGF-beta), that does not bind to its receptors [16, 17]. In vitro TGF-beta can be activated by passagere acidification, heating or by enzymatic mechanisms [18-20]. For nothing is known about the influence of antiprogestins on the TGF-beta production, we addressed our question to whether the progesterone antagonist onapristone can induce TGF-beta secretion in breast cancer cells in vitro and whether a possible induction correlates with the antiproliferative effect and the receptor status of the cells.

Materials and methods

Cells and cell culture

MCF7 and T-47D cells were obtained by the American Type Culture Collection (Maryland, U.S.A.). A549 and T-47D/x (a ER-/PR-
variant) were kindly provided by Dr. C. Knabbe (University of Hamburg, Germany). Prior to experiments all cell lines were cultured in RPMI-1640 medium (Seromed, Berlin) supplemented with 10% fetal bovine serum (FBS; Seromed, Berlin), 2 mM t-glutamine and 50 U penicillin/streptomycin in a 5% CO₂ atmosphere. In the cell lines T47-D, T47-D/x and MCF7 we investigated the proliferation (cell number) under basal, not estrogen-stimulated conditions on days 10 and 12 after passing: 0.5 x 10⁶ exponentially growing cells were plated into 75-cm² flasks (Becton Dickinson, Heidelberg, Germany), washed after 72 hours with phosphate-buffered saline and cultured in 30 ml medium supplemented with 10% FCS, which was treated with dextran-coated charcoal (FCS-DCC) for removal of steroidal agents. Onapristone (ZK 98.299, kindly provided by Schering, Berlin), dextranose or ethanol vehicle in 0.1% final concentration was added at concentrations of 10⁻⁹ M. Media (FCS-DCC) were routinely changed on days 3, 6, 9 and 12.

**Growth factors**

Human, recombinant [123]-Bolton Hunter labeled Transforming Growth Factor-ß1 with a specific activity of 3000-4500 Ci/mmol was obtained from Du Pont Dreieich, human natural TGF-beta1 derived from human platelets from Biermann, Bad Nauheim.

**Quantification of ER an PR**

The ER and PR concentrations were determined in those cells, in which the TGF-beta secretion was ascertained, using a monoclonal enzyme immunoassay (ER-EIA, PR-EIA Monoclonal Kit; Abbot, Wiesbaden, Germany) and expressed as fmol/mg protein.

**Preparation of conditioned media**

Serum-free conditioned medium (CM) was prepared as described earlier [11, 12] with some modifications. Near confluent monolayers of MCF7, T-47D or T-47D/x cells, grown for about 7 days in Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 5% sulfatase and charcoal-treated calf serum (FCS-CCS) [21] in the presence of different concentrations (10⁻⁹ M, 5 x 10⁻¹⁰ M, 10⁻¹¹ M) of onapristone, dextranose or ethanol vehicle alone in 0.1% final concentration, were washed twice with warm PBS and subsequently incubated in serum-free medium under continuous antihormonal treatment: 30 ml per petri dish DMEM with glutamine, 0.2 IU bovine insulin/ml, 5 μg human transferrin/ml, 2 μg bovine fibronectin/ml, 10 ng murine EGF/ml, 1 μM sodium selenite and 0.1 μM cupric sulfate (all but fibronectin [Biochrom] obtained from Sigma Deisenhofen, Germany). After 6 hours the CM was discarded and the cells again washed twice with PBS. After a further 48 hours incubation with fresh serum-free medium and with the drugs in concentrations mentioned above the CM was removed in 50 ml tubes, combined with 0.2% (v/v) aprotinin (20 μg/ml inhibitor units/ml), centrifuged at 800 g at 4 °C and finally clarified by passing through a 0.45 μm filter (Millipore Co. Bedford, MA). The filtrate was concentrated 12- to 28-fold in a Centricon microconcentrator (Amicon Co., Danvers, MA) with a molecular weight cutoff of 10 kD. The recovery of [125]TGF-beta1 added immediately after the collection was 40%. Cell monolayers were harvested with a PBS 0.05% trypsin/0.02% EDTA solution for protein quantification [22]. Acid activation experiments were carried out as described earlier [23]. Aliquots were acidified by the addition of HCl to a final concentration of 115 mM (pH 3.2) and incubated for 1 hour at 4 °C, subsequently reneutralized by addition of 1/40 volume of 1M NaHEPES (pH 7.4) and an appropriate volume of NaOH. In corresponding neutral aliquots, acid, base, and HEPES buffer were pre-mixed before addition to obtain an ionic strength identical to that of the transiently acidified aliquots.

**TGF-beta radioreceptor assay**

Total TGF-beta levels (LTGF-+ active TGF-beta) in the conditioned media and concentrations of active TGF-beta were determined in the TGF-beta Radioreceptor Assay described earlier [24] with some modifications. A549 cells were seeded in 24 well cluster plates, at 140,000 cells/well in DMEM supplemented with 10% FCS and grown for 18 hour in a 5% CO₂ atmosphere at 37 °C. Subsequently cells were washed 3 times with prewarmed binding buffer (DMEM, 1 mg/ml bovine serum albumin [BSA, Sigma] and 40 mM HEPES buffer [pH 7.4]). Binding buffer (200 μl/well) containing 20 pM [125]TGF-beta1 and serial dilutions of CM or unbleached TGF-beta1 (for standard curve) was added and allowed to bind 2 hours at 20 °C. Cells were then washed three times with ice cold Hank's balanced salt solution (Gibco) containing 1 mg/ml BSA. Cells were solubilized for 30 min. at 37 °C with 750 μl prewarmed Triton solution (20 mM HEPES, 1% Triton x-100, 10% glycerol). Radioactivity of an 600 μl aliquot was determined by a liquid scintillating system (Beckmann LS 5000). TGF-beta present in conditioned media samples was determined by comparison with a standard competition curve. TGF-beta concentrations in the CM were then normalized to the protein content of the producer monolayer.

**Statistical analysis**

Data are presented as the mean ± standard deviation for three to twelve experiments per group. Differences between control and treatment groups were determined by the Mann-Whitney Two Sample Test.

**Results**

**Steroid receptor status**

The analysis of the basal, not estrogen-stimulated hormone receptor status showed that T47-D and MCF7 were ER- and PR-positive, whereas T-47D/x proved to be ER- and PR-negative. Individual receptor concentrations are listed in Table 1.

**TGF-beta secretion** (Figure 1)

Onapristone stimulated the total TGF-beta secretion (LTGF-beta and active TGF-beta) of the T-47D cells on average 4.8-fold compared to untreated cells (146 ± 57 ng/mg protein, n = 4, vs. 30 ± 5 ng/mg protein, n = 12; P = 0.0045). All tested concentrations showed similar stimulatory effects. Secretion of active TGF-beta was increased only 2.9-fold on average (52 ± 22 ng/mg protein, n = 9, vs. 18 ± 2.6 ng/mg protein, n = 3; P = 0.016). Here the onapristone concentration 5 x 10⁻⁷ M showed to be a stimulatory optimum

**Table 1.** Estrogen (ER) and progesteronereceptor (PR) content of the tested cell lines.

<table>
<thead>
<tr>
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<th>ER</th>
<th>PR</th>
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<tr>
<td>T-47D</td>
<td>46 ± 11</td>
<td>456 ± 37</td>
</tr>
<tr>
<td>MCF7</td>
<td>15 ± 6</td>
<td>42 ± 20</td>
</tr>
<tr>
<td>T-47D/x</td>
<td>—</td>
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Figure 1. Hormonal control of TGF-β. Total (whole bar) and receptor-reactive (lower, unfilled part of bar) TGF-β in 48 hour-conditioned media from T-47D and MCF7 cells under long-term treatment (9 days) with the antiprogestin onapristone (Ona) and the antiestrogen droloxifene (Drol) compared to nontreated controls (Co). Data (mean ± SD) are normalized to the protein content of the producer monolayer. 4 to 12 experiments per group.

(4-fold stimulation) of the TGF-beta (active) secretion.

Droloxifene induced total TGF-beta secretion of the T-47D cells 4.2-fold (129 ± 45 ng/mg protein, n = 12, vs. 30 ± 5.4 ng/mg protein, n = 4; P = 0.005). Stimulation of active TGF-beta was of less extent: only 1.7-fold (marginally significant); however the stimulation of active TGF-beta by droloxifene seems to be dose dependent: 2.5-fold stimulation with a droloxifene concentration of 10⁻⁶ M and no stimulation with a concentration of 10⁻⁷ M.

Onapristone showed no TGF-beta induction in MCF7 cells (n = 5–6 per group).

Application of droloxifene to MCF7 cells, however, resulted in a significant 5.8-fold increase of total TGF-beta concentration in the conditioned media (19 ± 16 ng/mg protein, n = 9, vs. 3.2 ± 1.4 ng/mg protein n = 5; P = 0.046). The stimulatory effect seems to be dose dependent: highest with a droloxifene concentration of 10⁻⁶ M (9.5-fold) and no stimulation with a droloxifene concentration of 10⁻⁷ M. Secretion of active TGF-beta was not influenced.

In the T-47D/x cells TGF-beta levels were not increased neither by droloxifene nor by onapristone.

Cell growth

Onapristone (1 × 10⁻⁶ M) inhibited the growth of T-47D cells very effectively. Cell counts of the treated cells were about 65% (day 10: 2.32 ± 0.18) and 69% (day 13: 3.97 ± 0.1) lower than the counts of untreated cells (day 10: 6.72 ± 0.18, day 13: 12.73 ± 0.25). The treatment with droloxifene resulted in a growth inhibition of only 43% (day 10: 3.86 ± 0.04) and 45% (day 13: 6.99 ± 0.35), respectively (Figure 2a).

Growth of MCF7 cells was inhibited by both, onapristone and droloxifene. However, there was a greater reduction of cell growth using droloxifene compared to onapristone (70% vs. 26% on day 10; 39% vs. 15% on day 13; Figure 2b).

Growth of the T-47D/x cells was neither inhibited by onapristone nor by droloxifene.

Estrogen- and progesteronereceptor analysis (Figure 3)

Onapristone did not result in a significant change of the ER neither in T-47D nor in MCF7 cells. The PR, however, was dramatically downregulated in both cell lines. In T-47D the PR reduction compared to untreated cells was on average 93% (45 ± 26 fmol/mg protein vs. 456 ± 32 fmol/mg protein; P = 0.017) and in MCF7 cells 65% (22 ± 9 fmol/mg protein vs. 64 ± 4 fmol/mg protein; P = 0.001).

Droloxifene caused an increase of ER content in both ER positive cell lines, which was only in MCF7 cells statistically significant. Here the ER content was 4.9-fold higher compared to untreated cells (53 ± 32 vs. 11 ± 3 fmol/mg protein; P = 0.027). The PR were dramatically down regulated in both cell lines. In T-47D cells the reduction was 98% (11 ± 12 vs. 456 ± 0.18).
our experiments on onapristone showed no estrogenic activity, which could be a decisive advantage in the therapy of breast cancer, taking into account, that many breast carcinomas grow estrogen dependent. Bakker et al., however, observed a dramatic downregulation of PR content of rat mammary tumor tissue [33].

We observed an induction of total TGF-beta in the ER-positive T-47D and MCF7 cells by droloxifene, which are strongly growth inhibited by the antiestrogen. Those observations are congruent with the results found in literature, and support the postulate, formulated by Knabbe [12], that the antiproliferative effect of the antiestrogens is partly due to the ER-mediated ability of those substances, to induce the growth inhibitor TGF-beta [12]. In vivo tumor growth inhibition by antiestrogens can also be induced by stimulatory effects on TGF-beta secretion by stromal fibroblasts, which certainly contributes to the antitumor effect of antiestrogens [34]. The same could be true for antiprogestins, which remains to be investigated.

The dramatic down-regulation of the PR in both PR-positive cell lines by droloxifene indicates, that droloxifene has less estrogenic activity than the antiestrogen tamoxifen, which induced the PR in vitro and in vivo because of its estrogenic activity [35–37]. Less estrogenic activity of droloxifene compared to tamoxifen was suspected already earlier [38].

In summary, our data present evidence, that antiprogestins like onapristone exert their antiproliferative effect partly via induction of TGF-beta via the PR, like antiestrogens do via the ER.

Acknowledgments

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References


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