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The activation of an extracellular signal-regulated kinase by oestradiol interferes with the effects of trastuzumab on HER2 signalling in endometrial adenocarcinoma cell lines

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Abstract

Cellular response to oestradiol stimuli is mediated both by oestrogen receptor (ER) binding to oestrogen response elements (EREs) and by non-nuclear actions like activation of mitogen-activated protein kinase (MAPK) signal transduction. Therefore, oestradiol stimuli might be able to interfere with the action of antitumoral substances directed against receptor tyrosine kinase signalling. We investigated the effect of oestradiol on the inhibition of HER2 signalling by trastuzumab (Herceptin™) in two human endometrial adenocarcinoma cell lines. Activation of the extracellular signal-regulated kinase (ERK-1/2), a major mediator of HER2 signalling, was measured by means of western blotting experiments and ERE activation was determined in transient reporter-gene assays. In endometrial Ishikawa and HEC-1A adenocarcinoma cells, HER2 signalling was inhibited by trastuzumab only in the absence of oestradiol. We were able to demonstrate that oestradiol counteracted the inhibitory effects of trastuzumab by rapid phosphorylation of ERK-1/2, a kinase downstream of the HER2 receptor. The pure anti-oestrogen ICI 182,780 was able to restore both the trastuzumab-triggered inhibition of the ERK-1/2 pathway and the antiproliferative action of this substance in Ishikawa cells. Our data suggest that combinations of trastuzumab with anti-oestrogens may be effective in the treatment of endometrial cancers with a positive ER and HER2 receptor status.

Keywords: Endometrial adenocarcinoma; Oestradiol; Heregulin; Trastuzumab; Anti-oestrogen; Extracellular signal-regulated kinase

1. Introduction

Growth of human endometrial adenocarcinoma cells is regulated by steroids and peptide hormones [1]. Cellular effects are mediated by oestrogen receptors (ERs) and by receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) or HER2 (c-erbB-2). In endometrial cancer cells, aberrant expression of these receptors can be observed suggesting dysregulated control of cellular proliferation [2].

Overexpression of the *HER2* oncogene occurs in 15–20% of endometrial cancers and is associated with a poor survival [3]. Moderate expression of this gene can be observed in half of the cases [4,5]. The *HER2* gene encodes a 185 kD transmembrane receptor tyrosine kinase, which functions as a co-receptor for other members of the HER family. HER2 can be transactivated by secretory polypeptides like heregulin or be activated by its own overexpression. HER2 signalling is mediated by signal transduction cascades like the $p21^{Ras}$/mitogen-activated protein kinase (MAPK), S6 kinase and phospholipase C-γ pathways. HER2 signalling can be inhibited by trastuzumab (Herceptin™; Genentech, Inc, San Francisco, CA, USA), a substance also leading to HER2 receptor downregulation. This high-affinity, recombinant humanised anti-HER2 antibody has been used sucessfully in the treatment of patients with metastatic breast cancer that overexpress HER2 [6]. Besides HER2, also HER3 and HER4 receptors as well as peptide hormones like epidermal growth factor (EGF) or heregulins are expressed in normal endometrium, as well as in endometrial cancer [7]. This suggests the participation of HER-family receptors in the regulation of proliferation in this tissue.

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Therefore, HER2 may also be used as a target in the treatment of endometrial adenocarcinoma.

In the present study, we intended to investigate the effect of trastuzumab on the heregulin-β1-induced, HER2-mediated growth of endometrial cancer cell lines. In addition, we examined the influence of oestradiol on the efficacy of this antitumoral agent. This steroid is one of the major mitogenic signals in the endometrium, with the ability to activate ERs and also the $p21^{Ras}$/MAP kinase pathway [8,9]. Therefore, oestradiol activation of ERK-1/2 might interact with HER2 signalling. In this study, we tested the hypothesis, whether oestradiol is able to interfere with the inhibitory effect of trastuzumab on HER2 signalling in endometrial cancer cell lines by activation of kinases downstream of this receptor. For this purpose, we employed the ER-positive cell line, Ishikawa, representing a well-differentiated endometrial adenocarcinoma, and the ER-poor line, HEC-1A, derived from a poorly differentiated endometrial adenocarcinoma [10,11].

2. Materials and methods

2.1. Materials

Human recombinant heregulin-β1 was purchased from Oncogene Research Products (Boston, MA, USA), recombinant humanised anti-HER-2 antibody, trastuzumab (Herceptin™), was provided by Genentech (San Francisco, CA, USA). Water-soluble 17-β-oestradiol was obtained from Sigma (Deisenhofen, Germany), pure anti-oestrogen ICI 182,780 was purchased from Tocris (Bristol, UK).

Fetal calf serum (FCS) was purchased from GibcoBRL, phenol red-free Dulbecco's Modified Eagle Medium (DMEM) medium (with 1000 mg/l glucose and with L-glutamine) and Serum Replacement 2 (SR2) were obtained from Sigma (Germany).

Beta-Gal Detection Kit was purchased from Promega (Madison, USA), Cell Proliferation enzyme-linked immunosorbent assay (ELISA) Kit was from Roche (Mannheim, Germany). Great EscAPe SEAP (Secreted Alkaline Phosphatase) Detection Kit and Mercury Pathway Profiling System 2 containing vectors pSEAP2-Control and pERE-TA-SEAP were obtained from Clontech (Palo Alto, CA, USA).

2.2. Cell culture

Ishikawa and HEC-1A endometrial adenocarcinoma cells (obtained from the American Type Culture Collection, Manassas, VA, USA) were maintained in phenol red-free DMEM medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. For serum-free culture, both cells lines were cultured in DMEM containing the defined, oestradiol- and growth factor-free Serum Replacement 2 (SR2, Sigma). Both cell lines were cultured with 5% CO_2 at 37 °C.

2.3. Proliferation assays

Cells were suspended in phenol red-free DMEM containing 5% (v/v) steroid depleted charcoal-treated medium (SFCS) and seeded in 96-well plates ($3–5\times10^3$ cells/well). After 24 h, the medium was changed (0.5% (v/v) SFCS) and after 48 h, cells were washed and incubated in DMEM-SR2, followed by a treatment with the test substances in quadruplicates. After 72 h of treatment, cellular proliferation was quantified by measurement of relative cell numbers using the Cell Titer Cell Proliferation (MTS-) Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cell growth was expressed as a percentage of the serum-free medium control. The data obtained in three or four different experiments were pooled. After a Bartlett test had shown that the variances were homogenous, analysis of variance (ANOVA) was carried out. Then the data was analysed for statistical significance of differences between individual groups using the Newman–Keuls test and the Prism 2.0 Software (Graph Pad, San Diego, CA, USA), with statistical significance accepted at $P<0.05$.

2.4. Antibodies and western blotting analysis

Cells were lysed in RIPA buffer (1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS) in phosphate-buffered solution (PBS) containing aprotinin and sodium orthovanadate). Aliquots containing 10 μg protein were resolved by 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE), followed by electrotransfer to a nitrocellulose membrane. Immunodetection was carried out using ERK-1/2 (K-23) and pERK (E-4) antibodies (Santa Cruz Biotech, Santa Cruz, USA, both diluted 1:500) and c-neu (Ab-6) antibody (Calbiochem, Bad Soden, Germany), diluted 1:250 in PBS containing 5% skim milk (w/v), followed by horseradish peroxidase-conjugated secondary antibodies which were detected using the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). ERK-1/2 activation was defined as the sum of the phosphorylated ERK-1 and ERK-2 signal and was quantified in relation to total ERK-1/2 protein after measurement of the optical density using EasyWin software (Herolab, Wiesloch, Germany). The data obtained in three or four different experiments were pooled. After a Bartlett-test had shown that variances were homogenous, analysis of variance (ANOVA) was carried out. Then the data was analysed for statistical significance of differences between individual groups using the Newman–Keuls

test and the Prism 2.0 Software (Graph Pad, San Diego, CA, USA), with statistical significance accepted at $P<0.05$.

2.5. Transfections and reporter gene assays

Transient transfection assays of endometrial cell lines, HEC-1A and Ishikawa, were performed in 24-well plates using Lipofectamine PLUS reagent (Invitrogen). The day prior to the transfection, 1×10^5 cells per well were seeded in 5% SFCS, after 4 h the medium was changed (DMEM/SR2). Transfection was performed according to the manufacturer's instructions, using the vectors pSEAP2-control and pERE-TA-SEAP (Clontech, Palo Alto, CA, USA), both coding for the secreted alkaline phosphatase reporter gene, co-transfected with the vector pCMV-beta (Clontech) as an internal standard. Cells were treated with oestradiol (1–100 nM) or ICI 182,780 (100 nM) 3 h later. 48 h after the transfection, aliquots of the culture supernatants were subjected to the SEAP assay PhosphaLight (Applied Biosystems, Foster City, CA, USA) and measured in a Packard LumiCount luminometer. As an internal standard, beta-galactosidase (β-gal) activity in the adherent cells was determined using the luminometric GalactoLight Plus enzyme assay (Applied Biosystems) according to the manufacturer's instructions. Oestradiol-induced ERE activation was quantified as the ratio of SEAP and β-gal activity.

3. Results

3.1. Expression of HER2 and oestradiol response of both cell lines

In order to examine the HER2 expression in the endometrial cancer cell lines employed in the experiments, we performed a western blotting analysis of HEC-1A and Ishikawa cell lysates. We were able to demonstrate the expression of HER2 in both cell lines (Fig. 1a), with a moderate expression level if compared with the HER2-overexpressing ovarian cancer cell line, SK-OV-3 (data not shown). In order to examine the ER function in both cell lines, we tested the oestradiol-triggered activation of oestrogen response elements (EREs). Treatment with all tested oestradiol concentrations (from 1 to 100 nM) resulted in the transcriptional induction of a SEAP reporter gene under the control of a minimal promoter containing two EREs (vector pERE-TA-SEAP) in Ishikawa, but not in HEC-1A cells (Fig. 1b). This ERE induction in Ishikawa cells could be inhibited by cotreatment with the pure anti-oestrogen ICI 182,780 (100 nM). Oestradiol treatment for 72 h was able to increase the proliferation of Ishikawa cells (approximately 2.5-fold) and also of HEC-1A cells (approximately 1.5-fold) (Fig. 2). This proliferative effect of oestradiol could be inhibited by cotreatment with 100 nM ICI 182,780 in ERα/β-positive Ishikawa cells only.

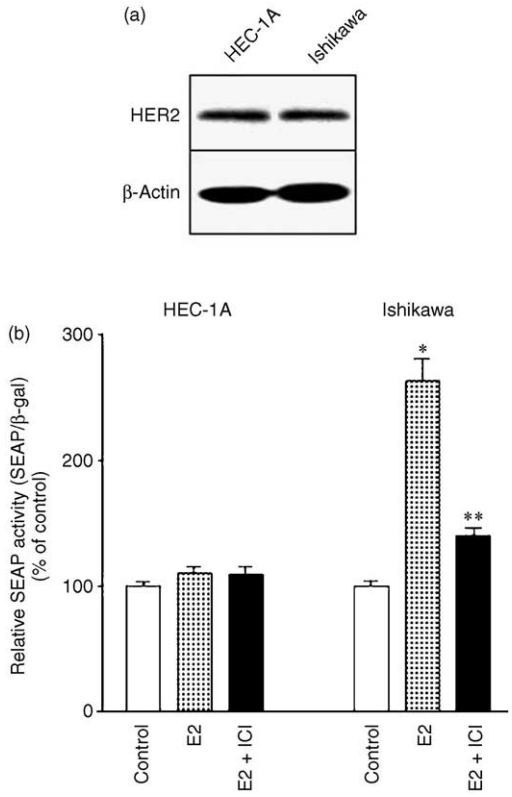

Fig. 1. HER2 expression and oestradiol response in HEC-1A and Ishikawa endometrial adenocarcinoma cells. (a) Western blotting analysis of HER2 expression in both cell lines. Detection of β-actin expression was used as a loading control. (b) Oestrogen response element (ERE) activation following oestradiol (E2) treatment and combined treatment with the anti-oestrogen ICI 182,780 (ICI). The ERE driven transcription of a Secreted Alkaline Phosphatase (SEAP) reporter gene was measured. Results were determined in quadruplicates in three separate experiments and expressed as means ±standard deviation (S.D.). *$P<0.05$ versus control; **$P<0.05$ versus E2.

3.2. Effect of trastuzumab on proliferation of endometrial adenocarcinoma cells

In the initial proliferation assays, we studied the effects of heregulin-β1, trastuzumab and oestradiol on the growth of the endometrial adenocarcinoma cell lines, HEC-1A and Ishikawa. Under serum-free culture conditions, heregulin-β1 concentrations of 1 to 100 nM were tested, but only concentrations of 10 and 100 nM led to a significant induction of cellular proliferation in both cell lines after 72 h of treatment. Simultaneous addition of 10 μg/ml trastuzumab resulted in an inhibition of the proliferative effect of heregulin-β1 in both cell lines cultured in serum- and oestradiol-free medium (Fig. 2). After treatment with trastuzumab alone as a

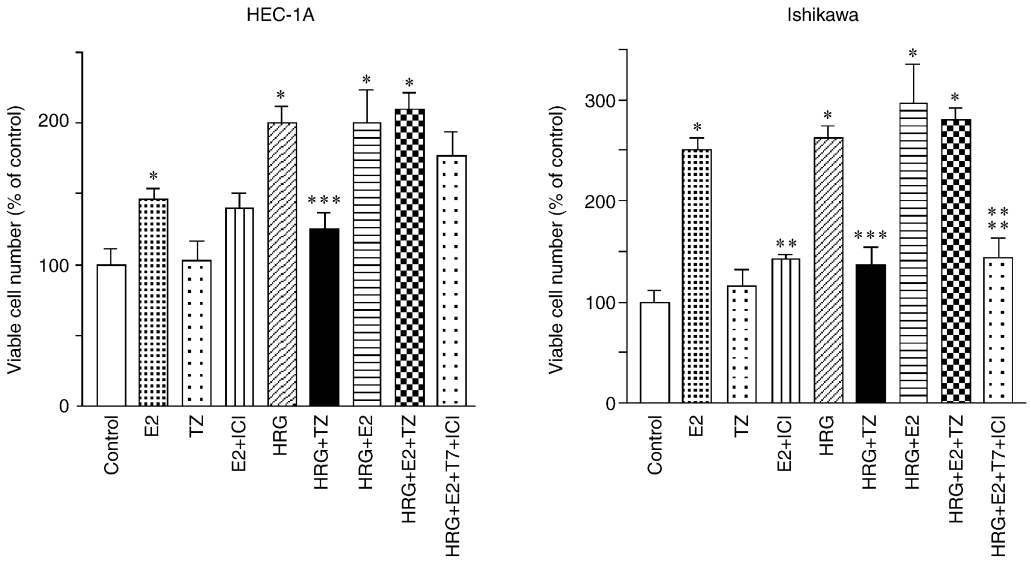

Fig. 2. Effects of oestradiol (E2) and ICI 182,780 (ICI) on heregulin-β1-(HRG)-induced and trastuzumab (TZ)-inhibited proliferation of HEC-1A and Ishikawa cells. Results were obtained from three separate experiments and expressed as means (% of control) ±S.D. *$P<0.05$ versus control; **$P<0.05$ versus E2; ***$P<0.05$ versus HRG; ****$P<0.05$ versus HRG + E2 + TZ.

control, no antiproliferative effect of this antibody could be observed in relation to the serum-free medium. In culture medium containing 10 nM oestradiol, enhanced proliferation of both cell lines was observed, an effect which was not additive with the heregulin-β1-induced proliferation. Under these culture conditions, no inhibition of heregulin-β1-induced proliferation by trastuzumab could be detected (Fig. 2). Experiments that were performed to reverse this effect of oestradiol on trastuzumab action by addition of the pure anti-oestrogen ICI 182,780 revealed different responses in the two cell lines. In Ishikawa cells, ICI 182,780 was able to diminish oestradiol-induced proliferation, and could also partially restore the inhibitory effect of trastuzumab on heregulin-β1-induced proliferation. In HEC-1A cells, ICI 182,780 showed no significant effects either on the proliferative action of oestradiol or on the effects of trastuzumab.

3.3. *Effect of heregulin-β1, oestradiol and trastuzumab on ERK-1/2 phosphorylation*

We intended to examine the molecular mechanism underlying the ability of oestradiol to diminish the inhibitory effect of trastuzumab on heregulin-β1-induced proliferation. We asked the question of whether oestradiol treatment could stimulate cellular proliferation counteracting the action of trastuzumab by ER-mediated ERE activation alone, or by direct interaction with a HER2 signalling pathway.

In order to detect downstream events triggered by activated HER2, we measured changes in the ERK-1/2 phosphorylation status, a kinase of the $p21^{Ras}$-pathway that has been shown to be a major mediator of HER2 signalling [12]. We examined ERK-1/2 activation in both cell lines in response to a treatment with heregulin-β1, trastuzumab and oestradiol. An activated serine/threonine kinase function of ERK-1/2 is associated with phosphorylation of tyrosine residues such as Tyr-204. Detection of the phosphorylation of Tyr-204 can therefore be used to measure the activation of the ERK-1/2 signal transduction pathway [13].

Stimulation of HEC-1A and Ishikawa cells with 10 and 100 nM heregulin-β1 resulted in a significant induction of ERK-1/2 phosphorylation up to approximately 2-fold in HEC-1A cells and approximately 1.8-fold in Ishikawa cells five minutes after treatment (Fig. 3). This heregulin-triggered activation of ERK-1/2 could be partially inhibited by a 24 h pretreatment with trastuzumab in both cell lines (Fig. 4). In relation to the medium control, trastuzumab alone did not affect changes in the ERK-1/2 phosphorylation status.

When examining the effects of oestradiol on MAP kinase activation in both endometrial cell lines, we succeeded in demonstrating the rapid activation of ERK-1/2 by this steroid. Five min after treatment, oestradiol induced a significant increase of ERK-1/2 phosphorylation, with a maximum activity at 100 nM (HEC-1A) or 10 nM oestradiol (Ishikawa) (Fig. 5). In experiments combining the treatment with oestradiol and heregulin-β1, no additive effects on ERK-1/2 phosphorylation were measured. A pretreatment with trastuzumab for 24 h was able to impair this heregulin-induced ERK-1/2 phosphorylation, an effect that was only observed in cells cultured under oestradiol-free conditions (Fig. 6).

In order to examine the role of ERα as a mediator of oestradiol-triggered rapid ERK-1/2 activation, we used the pure anti-oestrogen ICI 182,780, which turned out

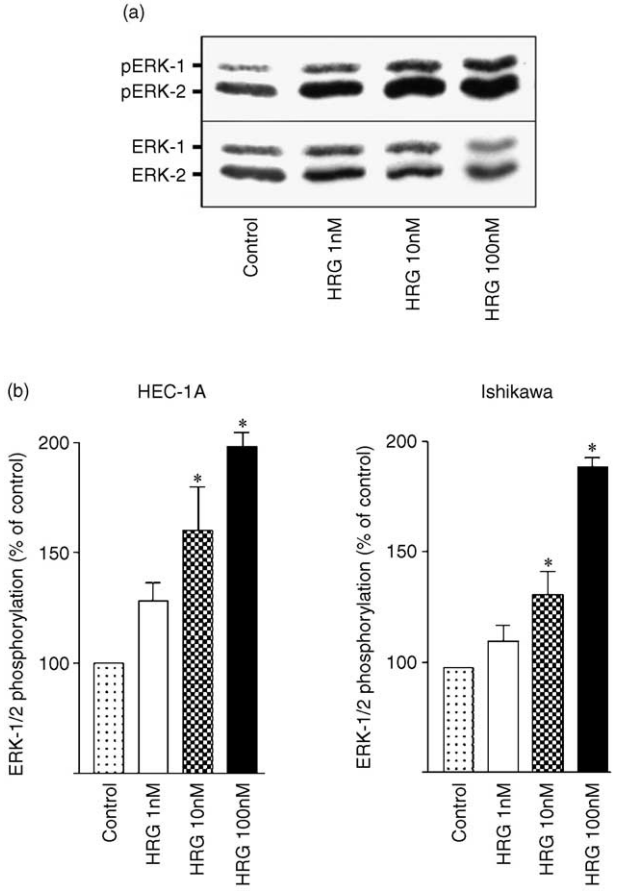

Fig. 3. Effect of heregulin-β1 (HRG) on the phosphorylation of extracellular regulated-kinase (ERK-1/2) (Tyr-204) in HEC-1A and Ishikawa cells. (a) Western blotting analysis of cell lysates from HEC-1A cells treated for 5 min with heregulin-β1 (1-100 nM). Using a phospho-specific ERK-1/2 antibody, two immunoreactive bands were detected, 44 kD ERK-1 and 42 kD ERK-2. As a loading control, an additional western blotting analysis using an antibody detecting total ERK-1/2 protein was performed. (b) Data from four separate western blotting experiments examining the heregulin-β1 effect on ERK-1/2 phosphorylation. Bands were quantified by densitometry (EasyWin, Herolab) and were expressed in percent of control as means ±S.D. $^{*}P<0.05$ versus control.

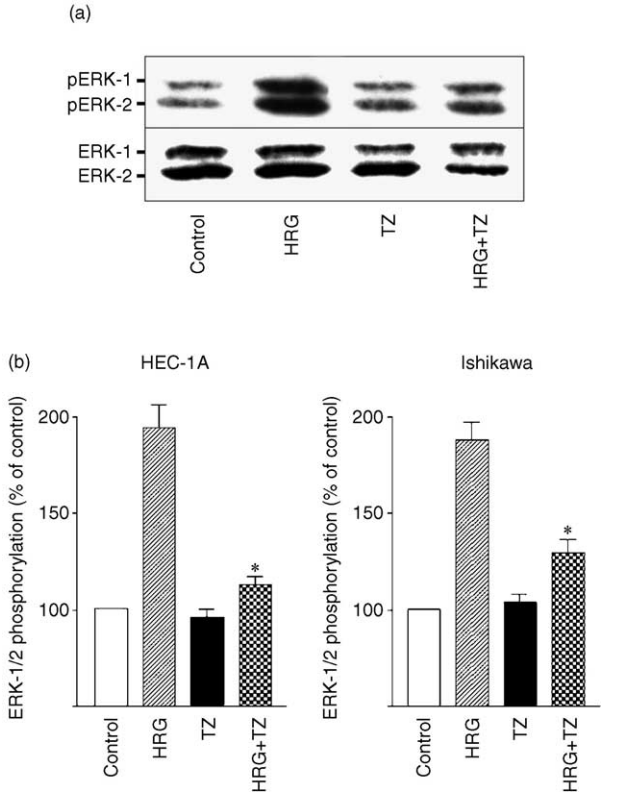

Fig. 4. Effect of trastuzumab on heregulin-β1-induced ERK-1/2 phosphorylation (Tyr-204). (a) Western blotting analysis of cell lysates from Ishikawa cells treated with heregulin-β1 (HRG, 100 nM) for 5 min or pretreated with trastuzumab (TZ, 10 μg/ml) for 24 h. As a loading control, an additional western blotting analysis using an antibody detecting total ERK-1/2 was performed. (b) Data from five separate western blotting experiments examining the trastuzumab effect on heregulin-β1-induced ERK-1/2 phosphorylation. Bands were quantified by densitometry (EasyWin, Herolab) and were expressed in percent of control as means ±S.D. $^{*}P<0.05$ versus HRG.

to inhibit the activation of ERK-1/2 by oestradiol stimulation in Ishikawa cells only. Moreover, in this cell line, the ERK-1/2 phosphorylation induced by co-treatment with oestradiol and heregulin-β1 was significantly inhibited by the combinatory use of ICI 182,780 and trastuzumab, but not by treatment with trastuzumab alone (Fig. 7).

4. Discussion

The results presented in this study demonstrate the inhibitory effect of trastuzumab on heregulin-β1-induced ERK-1/2 activation and proliferation of two endometrial cancer cell lines both expressing moderate levels of HER2 protein. In both cell lines, this effect was impaired by oestradiol, which was shown to counteract the action of trastuzumab by rapid activation of ERK-1/2, a kinase downstream of the HER2 receptor tyrosine kinase.

Today, trastuzumab is used for the therapy of breast cancer patients with HER2-overexpressing tumours. However, a phase II evaluation of trastuzumab in recurrent and advanced endometrial carcinomas is underway (GOG trial 181B).

Recently, it has been shown, that trastuzumab action can be defined not only by its effects on HER2-overexpressing cells, but also by its ability to impair HER2 signalling triggered by heregulins [14]. HER2 can be activated both by its own overexpression and by heregulin stimuli, and in breast cancer cell lines, HER2 activation is inhibited by trastuzumab irrespective of the cause of this activation. Because there is no existent cell culture model for HER2-overexpressing endometrial adenocarcinoma, we used heregulin-responsive, moderate HER2-positive endometrial cancer cell lines that

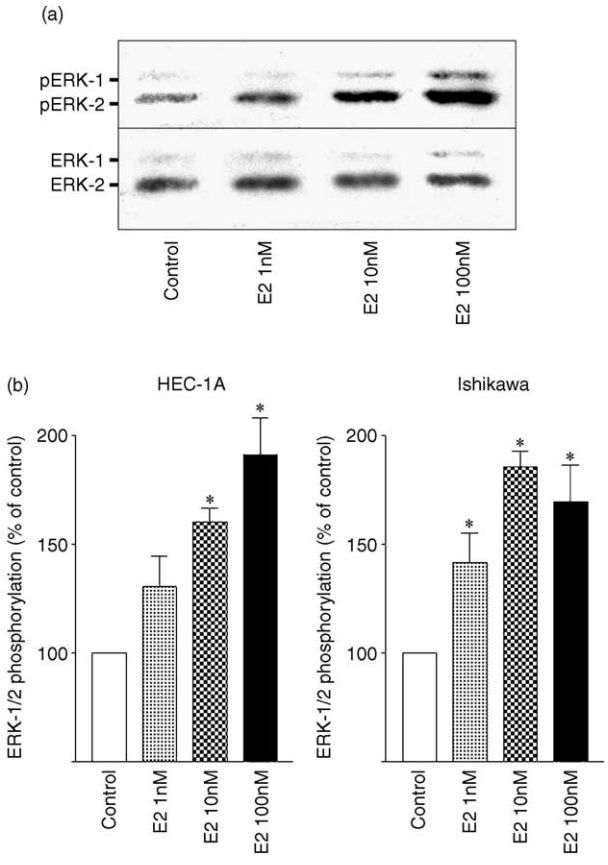

Fig. 5. Rapid effect of 17-β-oestradiol on ERK-1/2 phosphorylation (Tyr-204). (a) Western blotting analysis of cell lysates from HEC-1A cells treated with water-soluble oestradiol (E2, 1–100 nM) for 5 min. As a loading control, an additional western blotting analysis using an antibody detecting total ERK-1/2 was performed. (b) Data from four separate western blotting experiments examining the oestradiol effect on ERK-1/2 phosphorylation. Bands were quantified by densitometry (EasyWin, Herolab) and were expressed in percent of control as means ±S.D. *$P<0.05$ versus control.

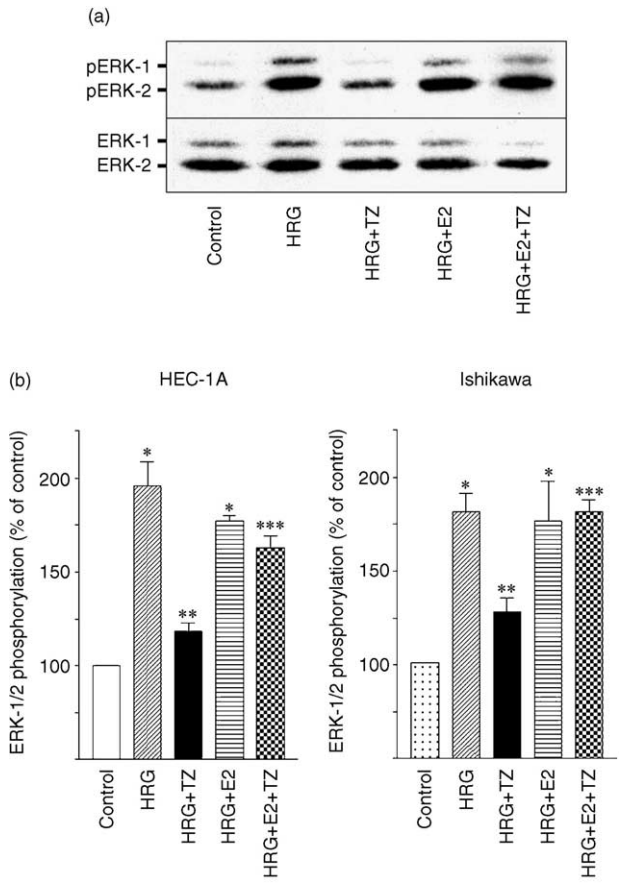

Fig. 6. Effect of 17-β-oestradiol on the inhibition of heregulin-β1-induced ERK-1/2 phosphorylation by trastuzumab. (a) Western blotting analysis of cell lysates from Ishikawa cells preincubated with trastuzumab (TZ, 10 μg/ml) for 24 h and then treated with combinations of heregulin-β1 (HRG, 100 nM) and oestradiol (E2, 10 nM) for 5 min. As a loading control, an additional western blotting analysis using an antibody detecting total ERK-1/2 was performed. (b) Data from six separate western blotting experiments examining the oestradiol effect (Ishikawa 10 nM, HEC-1A 100 nM oestradiol) on trastuzumab action. Bands were quantified by densitometry (EasyWin, Herolab) and were expressed in percent of control as means ±S.D. *$P<0.05$ versus control; **$P<0.05$ versus HRG; ***$P<0.05$ versus HRG + TZ.

provided a model for our studies of the trastuzumab effect on HER2 activation in carcinomas of this tissue.

Effects of *in vitro* heregulin-β1 treatment on breast cancer cell lines have been thoroughly examined, but relatively little is known about its action in endometrial tumour cells, though heregulin-β1 has been demonstrated to be expressed in endometrial cancers [7]. Heregulin-β1 has been shown to promote proliferation, motility and invasiveness of breast cancer cells with moderate or elevated HER2 expression [15,16]. Heregulin-β1 is a high affinity ligand of heterodimers of HER2 and HER3 or HER4, its binding results in tyrosine phosphorylation of the receptor molecules [17,18]. Heregulin transactivation of HER2 has the same Ras-dependent activating effect on ERK-1/2 as overexpression of this receptor tyrosine kinase [19]. Thus, with regard to ERK-1/2 activation, heregulin treatment is able to mimic the molecular effects of a HER2 overexpression. We used endometrial cancer cell lines with a moderate HER2 expression, since they provide a model for inducible HER2 signalling from very low levels (without heregulin stimulus) to levels comparable to the ones detectable in HER2-overexpressing cells.

In experiments with breast cancer-derived cell lines, heregulin-β1 has been shown to have a dual role, promoting mitogenesis as well as differentiation under certain conditions [20,21]. It has been shown, that heregulin is able to stimulate the proliferation of breast and ovarian cancer cell lines with a moderate HER2 status, as well as in overexpressing cell lines [22]. In breast cancer cell lines, previous studies revealed the ability of trastuzumab to enhance the antiproliferative effect of the pure anti-oestrogen ICI 182,780 in HER2- and ER-positive cells [23].

For this study, we employed the ERα/β-positive endometrial adenocarcinoma cell line, Ishikawa, and the

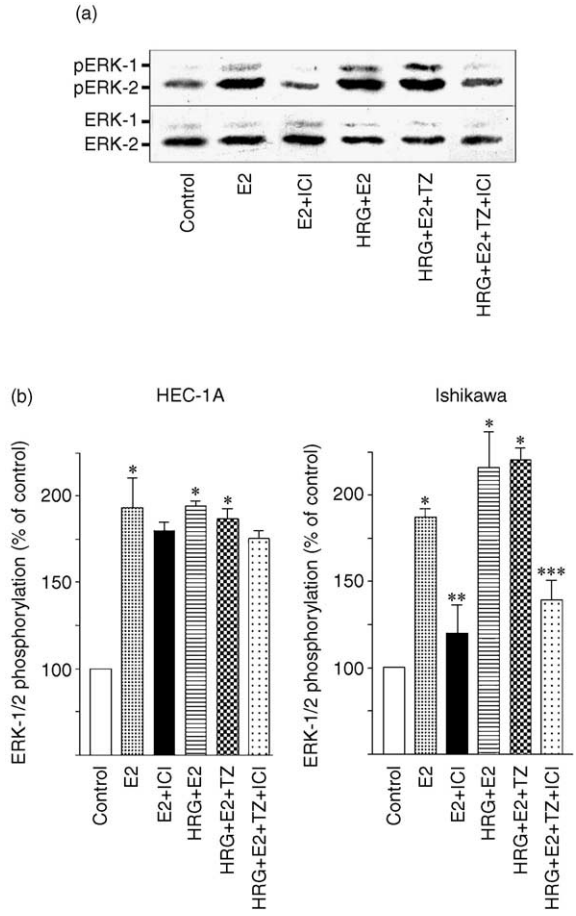

Fig. 7. Effect of ICI 182,780 on 17-β-oestradiol-induced ERK-1/2 phosphorylation. (a) Western blotting analysis of cell lysates from Ishikawa cells preincubated with trastuzumab (TZ, 10 μg/ml) for 24 h or without preincubation and then treated with combinations of heregulin-β1 (HRG, 100 nM), oestradiol (E2, 10 nM) and ICI 182,780 (ICI, 100 nM) for 5 min. As a loading control, an additional western blotting analysis using an antibody detecting total ERK-1/2 was performed. (b) Data from six separate western blotting experiments examining the effect of ICI 182,780 on oestradiol action (Ishikawa 10 nM, HEC-1A 100 nM oestradiol). Bands were quantified by densitometry (EasyWin, Herolab) and were expressed in percent of control as means ±S.D. $*P<0.05$ versus control; $**P<0.05$ versus E2; $***P<0.05$ versus HRG + TZ + E2.

ER-poor cell line, HEC-1A [24], in order to compare the effects of oestradiol on trastuzumab action in both cell lines. Analysing the activation of an ERE following oestradiol treatment by means of reporter gene studies, our data confirm the presence of functional ERs in the Ishikawa cell line. The absence of oestradiol effects on ERE activation in HEC-1A cells is consistent with previous findings classifying this cell line as ER-poor [10]. Thus, the moderate enhancement of cellular proliferation of HEC-1A cells after oestradiol treatment could be mediated by ERE-independent mitogenic signalling cascades, as we were able to show by the detection of a rapid ERK-1/2 activation induced by this steroid.

Our data demonstrates a mitogenic effect of a heregulin-β1 treatment on Ishikawa and HEC-1A endometrial adenocarcinoma cell lines, which both express moderate levels of HER2 protein. This proliferative effect of heregulin-β1 was accompanied by a rapid increase in ERK-1/2 activation. Both proliferation and p42/44 MAPK activation were blocked by MEK inhibitor PD98059 (data not shown) suggesting a role of ERK-1/2 as a mediator of the proliferative heregulin-β1 effect.

We were able to demonstrate a significant inhibition of heregulin-induced proliferation and ERK-1/2 activation by pretreatment with the recombinant humanised anti-HER2 antibody, trastuzumab, in both endometrial cancer cell lines when cultured under oestradiol-free conditions. In contrast to previous data obtained in breast cancer cell lines overexpressing HER2 or with a moderate HER2 status, in which trastuzumab was able to block heregulin-β1-induced signalling events irrespective of the presence of serum or oestradiol [25,26], in the endometrial cancer cells we observed a clear difference of trastuzumab action depending on the oestrogen environment. Addition of 17-β-oestradiol (10–100 nM) to the culture medium impaired the inhibitory effect of trastuzumab on heregulin-β1 action. The possibility of the involvement of classical oestradiol signalling mechanisms like the transcriptional induction of growth factor expression leading to an autocrine stimulation of receptor tyrosine kinases followed by activation of the p42/44 MAP kinase pathway is ruled out by the short time period between the oestradiol stimulus and ERK-1/2 activation. Our data suggest that this effect of oestradiol resulted from non-nuclear actions, like activation of cell membrane ERs [27] or binding to G-protein coupled receptors [28,29] leading to activation of ERK-1/2, the kinase also mediating HER2 signalling.

Both oestradiol-induced ERK-1/2 activation and cellular proliferation were blocked by the pure anti-oestrogen ICI 182,780 in the Ishikawa cell line, which expresses ERα and β. Previous studies on rat fibroblasts transfected with ERα- or ERβ-cDNA demonstrated the ability of the pure anti-oestrogen ICI 182,780 to inhibit the oestradiol-induced MAPK activation mediated by ERα, but not by ERβ [30]. Therefore, it is tempting to propose ERα as the main mediator of the non-genomic oestradiol effect in Ishikawa cells. In the ER-poor HEC-1A cell line, oestradiol also activated both ERK-1/2 enzyme and cellular proliferation, but moderately. Since neither ERE activation nor ICI 182,780 effects could be detected in the HEC-1A cells, our data suggest that the actions of oestradiol on cellular proliferation and ERK-1/2 activation were not mediated by the actions of ERα in this cell line. Since oestradiol triggered both reactions in Ishikawa as well as in HEC-1A cells, these effects seem to be mediated by two different non-nuclear mechanisms, which are ERα-dependent in Ishikawa and ERα-independent in HEC-1A cells.

Our data demonstrate the ability of trastuzumab to inhibit HER2 activation in endometrial adenocarcinoma cell lines cultured in an oestradiol-free environment. In the ERα/β-positive cell line, Ishikawa, oestradiol was able to counteract the inhibiting effects of trastuzumab on heregulin-β1 response in an ER-dependent manner, whereas in HEC-1A cells, the same oestradiol effect was observed independently of ER-mediated ERE activation. We demonstrated, that the pure anti-oestrogen ICI 182,780 was able to restore the action of trastuzuma exclusively in the ERα/β-positive Ishikawa endometrial cancer cells cultured with oestradiol. This effect of ICI 182,780 was due to its inhibition of oestradiol-triggered activation of ERK-1/2, a kinase downstream of the HER2 receptor tyrosine kinase. These findings suggest that combined treatment with trastuzumab and ICI 182,780 may be useful for the treatment of patients with endometrial cancer expressing both ERs and HER2.

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