High levels of oestrogen receptor- α in tumorigenesis: inhibition of cell growth and angiogenic factors

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Abstract. We previously found that the stable overexpression of oestrogen receptor- α in the human endothelial cell line ECV304* inhibits its growth *in vitro*, and that this inhibition is possibly mediated through a down-regulation of the vasoactive agents endothelin-1 and vascular endothelial growth factor. Here we show an *in vivo* growth-inhibitory effect of oestrogen receptor- α overexpression in tumours initiated in nude mice from the same clone of ECV304. In addition, we show that this growth inhibition is accompanied by an $\alpha_v\beta_3$ -mediated inhibition of cell migration *in vitro*, and a down-regulation of the integrin $\alpha_v\beta_3$, vascular endothelial growth factor and vascularization *in vivo*. The levels of vascular endothelial growth factor and integrin $\alpha_v\beta_3$, through their effect on cell growth and migration, contribute to the process of angiogenesis and to the pathogenesis of atherosclerosis and cancer. The results shown here demonstrate that a higher level of oestrogen receptor- α in the cell, through its effect on certain angiogenic factors, may play a role in the control of angiogenesis.

INTRODUCTION

Oestrogen is considered to be protective against atherosclerosis through an antiproliferative effect on vascular cells (Karas *et al.* 1993; Mendelsohn & Karas 1994; Morey *et al.* 1997). Similarly, the presence of oestrogen receptor (OR) is known to be favourable in the prognosis and treatment of breast (Aamdal *et al.* 1984), ovarian (Geisler *et al.* 1995) and endometrial cancers (Martin *et al.* 1983). In certain cell lines, a high level of OR- α has been reported to inhibit proliferation, metastasis and invasiveness (Garcia *et al.* 1982; Osborne *et al.* 1985; Price *et al.* 1992). Angiogenic cytokines such as vascular endothelial growth factor (VEGF) influence vascular cell growth.

*Since the completion of this work ATCC have re-characterized the ECV304 cell line as a derivative of the human bladder cell line T24. This change, however, does not affect the interpretation of the data presented in this paper.

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These cytokines also influence the expression of adhesion molecules, such as the integrin $\alpha_v\beta_3$, which in turn contributes to angiogenesis. By influencing angiogenesis, these angiogenic factors affect the growth of certain types of cancer, and through the same effect may also contribute to the pathogenesis of atherosclerosis. In light of the recent developments in the role of angiogenesis in tumour progression (Hanahan & Folkman 1996), the present work was designed to evaluate the hypothesis that oestrogen/OR- α inhibits *in vivo* cell growth by modulating the levels of certain growth-regulating factors in the vasculature. We have previously reported the development of a clone from the ECV304 cell line that was stably transfected with an OR- α cDNA and over-expressed the receptor at a level 10-fold higher than the parent cells (Ali *et al.* 1999). We also showed that this OR- α overexpression inhibited the *in vitro* growth (Ali *et al.* 1997, 1999), endothelin-1 (Ali *et al.* 1999) and VEGF (Mohamed *et al.* 1998) levels in ECV304 cells. Now we have used the same OR- α -overexpressing clone of ECV304 to initiate tumours *in vivo*. The studies presented here show that the overexpression of OR- α inhibits the growth of tumours initiated *in vivo* from ECV304 cells. We also demonstrate that this inhibition of tumour growth by high levels of OR- α may involve a down-regulation of the angiogenic factors $\alpha_v\beta_3$ and VEGF.

MATERIALS AND METHODS

Athymic mice

The 3-week-old nude female mouse strain Tac:Cr:(NCr)-nufBR was purchased from Taconic (Germantown, NY, USA). The animals were housed in an aseptic environment under controlled conditions of light and humidity and received food and water *ad libitum*. Animals were allowed to acclimate to the new environment for a week before ovariectomy or sham operation. For the week following the surgery, animals were kept on tetracycline water. The success of ovariectomy was ascertained by the confirmation of dioestrous cycle in a microscopic examination of vaginal swabs. The blood oestrogen levels in the ovariectomized, sham-operated and intact animals were confirmed by a radio-immunoassay.

Inoculation of athymic mice

The cells were routinely grown in Medium 199 (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA). On the day of inoculation, cells were trypsinized and resuspended in matrigel (from Fisher Scientific Co., Pittsburgh, PA, USA) at 1 million cells per 100 μ L. Animals were inoculated with 100 μ L of this suspension in each flank. Tumours were allowed to grow and their dimensions were measured weekly. Tumour weight was determined as follows (Osborne *et al.* 1985; Kubota *et al.* 1995):

estimated tumour weight (mg) = [longest diameter (mm) $\times$ (shortest diameter (mm))²]/2.

Treatment

One week after the ovariectomy, animals were inoculated with the cell suspension and implanted with a 60-day time release pellet of 17- β -oestradiol (E2), tamoxifen (TAM), 4-hydroxytamoxifen (OHT) or a placebo (Innovative Research of America, IRA, Sarasota, FL, USA). OHT was obtained from Sigma Chemicals (St. Louis, MO, USA) and was custom-prepared into 60-day time release pellets at IRA. A 12-gauge trochar was used to insert the pellet subcutaneously in the back of the animal's neck. Sixty days later, if the animal was still under observation, it was implanted with a fresh pellet.

The animal protocols described above were approved by the Animal Research Committee at the State University of New York at Buffalo. All experiments on the animals were performed while observing the NIH-endorsed guidelines.

Immunostaining for VEGF and integrin $\alpha_v\beta_3$

At the end of each experiment, the animals growing tumours were euthanized, and their tumours were excised and preserved in formalin until use. The tumours were embedded in paraffin, sectioned and stained for either integrin $\alpha_v\beta_3$ (using the anti- $\alpha_v\beta_3$ LM609 from R & D Systems, Inc., Minneapolis, MN) or for VEGF (using an anti-VEGF antibody from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The stained slides were then scored for the intensity of stain against the background of their respective negative controls stained in a similar fashion, except that the antibody was replaced with pre-immune serum. The intensity of stain thus measured, on a scale of 1–6 (6 being the highest intensity observed), was expressed as the level of VEGF or the integrin $\alpha_v\beta_3$ in tumours.

Staining for vascularization

The slides were prepared as for VEGF and $\alpha_v\beta_3$ staining. The staining for vascularization was performed using Verhoeff's elastic stain method (Carson 1990). Briefly, the slides were stained using a haematoxylin-ferric-chloride-iodine solution, which makes the vascular elastic fibres and the cell nuclei appear black. The slides were then scored for the magnitude of black-stained fibres, which was expressed, on a scale of 1–5 (5 being the highest magnitude observed), as the level of vascularization in tumours.

Cellular migration assay

The cells were trypsinized and resuspended in serum-free medium. Equal numbers of cells, with or without pre-treatment with the anti- $\alpha_v\beta_3$ antibody LM609, $\alpha_v\beta_3$ inhibitor XT199, or E2, were incubated onto an 8 μ m membrane. The cells were allowed to migrate overnight across the membrane and towards the chamber containing the chemokine fibronectin, vitronectin or osteopontin. The next day, the non-migrated cells were washed away and the membranes, containing the adherent, migrated cells, were treated with the fluorescent reagent rhodamine phalloidin. The filter was air-dried and the number of migrated cells was measured by measuring the fluorescence at 530 excitation and 590 emission.

RESULTS

Effect of OR overexpression on tumour growth

As described previously (Ali *et al.* 1999), to develop the cell line stably overexpressing the OR- α , ECV304 cells were cotransfected with a G418-resistance-conferring plasmid and an OR- α -expressing vector. The OR-overexpressing clones were selected for G418 resistance and then screened for high OR- α expression. All the subsequent experiments, including the ones presented here, were then performed using the parent ECV304 cells, OR-overexpressing clone of ECV304 (ECV-OR) and a control-transfected clone of ECV304 (ECV-NON). ECV-NON was selected during the screening for ECV-OR. ECV-NON was found to be G418-resistant but expressed parental levels of OR. Having gone through the same transfection process, ECV-NON was deemed a logical control against the phenotypic changes arising from the transfection protocol alone. In addition, ECV-NON was also a control against clone-dependent artefacts.

Figure 1. Effect of oestradiol and anti-oestrogens on *in vivo* growth of tumours initiated from parent (open bars), OR-transfected (solid bars) or control-transfected (hatched bars) ECV304 cells. (a) Three-week-old female nude mice, strain Tac:Cr:(NCr)-nufBR, were implanted with 1×10^6 cells in each flank. Tumours were allowed to grow and their weights were determined weekly. Animals were either ovariectomized (OVX) or sham-operated (SHAM). Some groups of the OVX animals were subcutaneously implanted with time-release pellets of 0.15 mg or 1.5 mg oestradiol (E2). The results are expressed as mean $\pm$ SE ($n \geq 8$). (b) Groups of OVX nude animals were also implanted with 1.5 mg time-release pellets of tamoxifen (TAM), 4-hydroxytamoxifen (OHT) or a placebo. The results are expressed as mean $\pm$ SE ($n \geq 8$).

To establish a baseline, each of the cell lines, i.e. ECV304, ECV-OR and ECV-NON, was inoculated into the flanks of ovariectomized (OVX) or sham-operated (SHAM) nude female mice. No visible tumours formed from any of the cell lines in either SHAM or OVX animals observed over a period of 5 weeks (Fig. 1a).

OVX animals inoculated with ECV304, ECV-OR and ECV-NON cells were implanted with 0.15 mg E2 pellets, which were formulated to allow a blood oestradiol level of 50–75 pg/ml (3-fold higher than the physiological E2 level in the nude mouse strain used). This group formed palpable tumours within 3 weeks (Fig. 1a). The tumour growth was more aggressive in mice inoculated with parent ECV304 and control ECV-NON cells, whereas it was significantly ($P \leq 0.01$, at week 3 and onwards) slower in the case of OR-transfected ECV-OR cells. When mice were implanted with 1.5 mg E2 pellets (allowing blood E2 levels of > 900 pg/ml), a considerable increase in tumour size was observed in the case of ECV304 and ECV-NON but no change was apparent in tumours from ECV-OR.

In all groups, therefore, oestradiol, at levels higher than physiological, stimulated tumour growth. In contrast, OR was found to be inhibitory to the growth of these tumours.

Ovariectomized mice implanted with 1.5 mg TAM or OHT pellets (allowing blood levels of 3–4 ng/ml in each case) developed palpable tumours from ECV304 as well as from ECV-OR cells within three weeks. ECV-OR cells, however, formed significantly ($P < 0.05$, at week 4 and onwards) smaller tumours in each case (Fig. 1b). Moreover, the tumours formed in these groups were remarkably smaller than the tumours in the corresponding groups implanted with E2 pellets. This indicated that the oestrogenic effect required for tumour formation was weaker in the case of anti-oestrogens than for E2.

Figure 2. $\alpha_v\beta_3$ -mediated migration in parent (open bars), OR-transfected (solid bars) and control-transfected (hatched bars) ECV304 cells. Equal numbers of cells were incubated onto an 8 μ m membrane. The cells were incubated either with (+609) or without (CONT) pre-treatment with the anti- $\alpha_v\beta_3$ antibody LM609, or in the presence of 10^{-8} M oestradiol (+E2). The cells were allowed to migrate overnight across the membrane and towards the chamber containing vitronectin. The next day, migrated cells on the membrane were measured in a fluorimetric assay. The results (mean $\pm$ SE) are representative of one of three separate experiments.

In these groups, therefore, anti-oestrogens were found to be stimulatory to tumour growth, but to a lesser extent than oestradiol. The OR overexpression was found to be consistently inhibitory to cell growth.

Effect on *in vitro* $\alpha_v\beta_3$ -mediated cell migration

$\alpha_v\beta_3$ is the most promiscuous member of the integrin family. It mediates cellular adhesion to several cell matrix proteins, including vitronectin, fibrinogen and osteopontin (Cheresh 1987; Leavesley *et al.* 1992). The cell migration assays for the integrin $\alpha_v\beta_3$ were therefore performed using human vitronectin, osteopontin and fibronectin as chemokines. Furthermore, to confirm whether or not this migration was $\alpha_v\beta_3$ -mediated, cells were tested either with or without pre-treatment with an anti- $\alpha_v\beta_3$ antibody.

As compared to the parent ECV304 cells, a significant ($P < 0.05$) decrease in cell migration was observed in OR-transfected cells using vitronectin matrix (Fig. 2). When the cells were pre-treated with anti- $\alpha_v\beta_3$ antibody (Fig. 2) or $\alpha_v\beta_3$ inhibitor XT199 (data not shown), the cell migration was significantly ($P < 0.5$) reduced in parent as well as OR-transfected cells – showing the migration to be $\alpha_v\beta_3$ -mediated. Qualitatively, similar results were observed with fibronectin and osteopontin matrices as well (data not shown). In the parent ECV304 cells, incubation in the presence of 10^{-8} M E2 also inhibited cell migration (Fig. 2) with vitronectin matrix. OR-transfected cells showed no change in their migration with or without E2. A similar trend was observed when the same concentration of E2 was used with fibronectin and osteopontin matrices (data not shown).

The effect of oestradiol as well as that of the OR-overexpression was therefore inhibitory on the migration of ECV304 cells.

Effects on the integrin $\alpha_v\beta_3$, VEGF and vascularization in tumours

To examine the levels of $\alpha_v\beta_3$ and VEGF, immunostaining with, respectively, an anti- $\alpha_v\beta_3$ antibody or an anti-VEGF antibody was performed on sections from tumours initiated in nude mice with parent and OR-transfected ECV304 cells. Slides from these tumours were also stained with a haematoxylin-ferric-chloride-iodine solution to examine the extent of vascularization. These tumours were excised from the animals that were implanted with the 0.15 mg E2 pellets. The tumours initiated from the OR-transfected cells showed a significant ($P \leq 0.05$) down-regulation of the integrin $\alpha_v\beta_3$ (Fig. 3a,b) and VEGF (Fig. 4a,b). As anticipated, the magnitude of vascularization was also found to be decreased in the OR-overexpressing tumours (Fig. 5a,b).

Figure 3. Levels of integrin $\alpha_v\beta_3$ in tumours initiated from parent (ECV304, open bars) and OR-transfected (ECV-OR, solid bars) ECV304 cells. (a) Formalin-preserved tumour samples were embedded in paraffin, sectioned, and stained using the anti- $\alpha_v\beta_3$ antibody LM609. The slides were scored for the intensity of positive stain against a background of their respective negative controls, which were stained similarly except that the anti- $\alpha_v\beta_3$ antibody was replaced with pre-immune serum. The size of the field of vision and the magnification were the same for all slides. The results, mean $\pm$ SE ($n \geq 8$), are expressed as the $\alpha_v\beta_3$ levels in tumours. (b) Representative slides of positive control (human kidney tissue) (left) tumours initiated from OR-transfected (middle) and parent (right) ECV304 cells. The $\alpha_v\beta_3$ -positive areas on the slides appear brown against a blue background.

Figure 4. Levels of VEGF in tumours initiated from parent (ECV304, open bars) and OR-transfected (ECV-OR, solid bars) ECV304 cells. (a) Tumour samples were prepared as in Fig. 4 and stained with an anti-VEGF antibody. The slides were scored as in Fig. 4 using similar negative controls. The size of the field of vision and the magnification were the same for all slides. The results, mean $\pm$ SE ($n \geq 8$), are expressed as the VEGF level in the tumours. (b) Representative slides of positive control (human tonsil tissue) (left) tumour initiated from OR-transfected (middle) and parent (right) ECV304 cells. The VEGF-positive areas on the slides appear brown against a blue background.

Figure 5. Vascularization in tumours initiated from parent (ECV304, open bars) and OR-transfected (ECV-OR, solid bars) ECV304 cells. (a) The slides were prepared as in Fig. 4 and stained using Verhoeff's elastic stain method and scored for the magnitude of black-stained fibres. The size of the field of vision and the magnification were the same for all slides. The results, mean $\pm$ SE ($n \geq 8$), are expressed as the level of vascularization in the tumours. (b) Representative slides of positive control (human aorta tissue) (left) tumours initiated from OR-transfected (middle) and parent (right) ECV304 cells. The extent of vascularization in the slides is observed as the black-stained elastic fibres.

In the tumours initiated from ECV304 cells, therefore, the OR-overexpression was found to inhibit the expression of the integrin $\alpha_v\beta_3$, VEGF and the degree of vascularization.

DISCUSSION

We found that the overexpression of OR- α caused growth inhibition of the tumours initiated from ECV304 cells. In oestrogen-deprived ovariectomized mice as well as in sham-operated animals, no tumour growth was observed from OR-transfected or parent cells. Only when ovariectomized mice were administered oestradiol to three times their physiological levels was the growth of tumours observed from parent, ECV-OR and ECV-NON cells. The growth of tumours was, however, significantly ($P = 0.008$ on day 8 of induction with oestradiol; Fig. 1a) slower from the OR-transfected clones. Similar results were obtained when TAM or OHT was used instead of oestradiol. The fact that no tumour growth was observed in sham-operated animals indicated that for their *in vivo* growth, ECV304 cells required a higher level of E2 than the normal physiological level in the nude mouse. Since the cell line used here is of human origin, it is quite comprehensible that the E2 blood levels in mice were not strong enough to elicit an oestrogenic effect in these cells.

These data indicate that oestrogen was not only stimulatory to the growth of these tumours but appeared to be a *requirement* for any growth to occur. The higher levels of oestrogen receptor, on the other hand, inhibit this growth. Our most significant observations were therefore finding consistently (1) a dissociation between the action of oestrogen receptor and its ligands (E2, TAM and OHT), and (2) an inhibitory action of OR- α on the *in vivo* growth of ECV304 cells.

The results presented here agree with our *in vitro* growth studies with ECV304 where the same OR-overexpressing clone ECV-OR was found to grow at a slower rate than the parent cells (Ali *et al.* 1999). In our preliminary studies, this growth-inhibition by OR- α overexpression was observed in other OR-overexpressing clones as well (data not shown): the growth-inhibitory effect of OR was therefore not a clone-dependent phenomenon. Moreover, our initial *in vivo* studies with the OR-overexpressing clones using several different strains of nude mice also showed consistency (data not shown), therefore demonstrating that the *in vivo* effect of OR overexpression was also independent of the mouse strain used. Both our earlier (Ali *et al.* 1997, 1999) *in vitro* and present *in vivo* studies consistently revealed a growth-inhibitory effect of OR- α overexpression on ECV304 cells. There was, however, a contrast between the *in vitro* and *in vivo* effect of oestradiol on ECV-OR cells, inhibitory in the former but stimulatory in the latter case. In growth studies, discordance between *in vitro* and *in vivo* oestrogen effects has been observed earlier with other cell lines (Langdon *et al.* 1993). These differences in the *in vitro* and *in vivo* results possibly reflect the interaction of OR with non-estrogenic ligands and/or oestrogen/androgen metabolites that may be effective only *in vivo*. OR is known to be transcriptionally activated by androgens and other non-estrogenic hormones (Zava & McGuire 1978a,b; Janne 1990; Smith 1998). Furthermore, oestrogen-response elements that are activated by metabolites of 17β -oestradiol bound to OR have recently been identified (Yang *et al.* 1996). It is therefore possible that the presence of excessive levels of OR in our system led to its binding to non-estrogenic hormones and/or androgen/oestrogen metabolites, resulting in an effect different from what is normally conferred by oestrogen in these cells.

Rapidly dividing cells need a continuously expanding network of blood-supplying vessels. The process of angiogenesis is therefore of crucial significance and can be the limiting factor in the pathogenesis of cancer, atherosclerosis, etc. Two key regulators of angiogenesis are cytokines

and adhesion molecules such as VEGF and the integrin $\alpha_v\beta_3$, respectively. There appears to be a correlation between VEGF and $\alpha_v\beta_3$. VEGF can up-regulate the levels of $\alpha_v\beta_3$ (Senger *et al.* 1997; Suzuma *et al.* 1998), and angiogenesis induced by VEGF can be blocked by the anti- $\alpha_v\beta_3$ antibody LM609 (Friedlander *et al.* 1995; Varner 1997). Taken together, these findings provide evidence that VEGF and $\alpha_v\beta_3$ may act co-operatively in regulating cell adhesion and migration, leading to the modulation of angiogenesis. To find out whether the oestrogen receptor-mediated growth effect in our tumours involved these angiogenic parameters, we studied the changes in the levels of VEGF and $\alpha_v\beta_3$ subsequent to OR- α overexpression. The effect on cell migration was also studied to gain further insight into the role of OR in the control of angiogenesis.

In cultured ECV304 cells, the overexpression of OR was found to inhibit VEGF (previous data, Ali *et al.* 1999) as well as the $\alpha_v\beta_3$ -mediated cell migration. In addition, tumours initiated from the OR-overexpressing cells showed a significant ($P \leq 0.05$) down-regulation of VEGF, integrin $\alpha_v\beta_3$ and vascularization. Taken together, these results indicated that the inhibition of proliferation and cell migration by OR in ECV304 cells may occur through an inhibition of growth-stimulating factors such as VEGF and the integrin $\alpha_v\beta_3$. The cumulative effect of the inhibition of these factors was possibly observed as a decrease in the level of vascularization and an inhibition of growth in the OR-overexpressing tumours. Since VEGF can stimulate $\alpha_v\beta_3$ expression (Senger *et al.* 1997; Suzuma *et al.* 1998), it is likely that the down-regulation of $\alpha_v\beta_3$ -mediated migration *in vitro*, and of $\alpha_v\beta_3$ levels *in vivo* by OR- α overexpression, occurs through inhibition of VEGF by OR.

In conclusion, the work presented here shows that a high cellular level of OR- α inhibits *in vivo* tumour formation, cell migration and vascularization. We also demonstrated that an OR- α -mediated down-regulation of VEGF and of the integrin $\alpha_v\beta_3$ may be responsible for the inhibition of angiogenesis observed here.

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