Functionality of the Progesterone Receptor in Ovarian Cancer and Its Regulation by Estrogen

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ABSTRACT
Here, we sought to obtain evidence that the progesterone receptor (PR) may be functional in ovarian cancer and regulated by estrogen. Megestrol acetate inhibited growth of the PR-positive PE04 ovarian carcinoma xenograft but not the PR-negative HOX 60 xenograft. PR concentration was higher in early-stage (I/II) tumors than in advanced-stage (III/IV) tumors (P = 0.007) and in tumors of endometrioid histology compared to other carcinoma subtypes (P = 0.009). Patients with a tumor PR concentration of >40 fmol/mg protein had significantly improved survival over those patients whose tumors contained <40 fmol/mg (P = 0.0007; log-rank).

Evidence of PR regulation by estrogen was obtained by endocrine manipulation of the PE04 xenograft. PR content of PE04 xenografts fell from 145 to 7 fmol/mg protein in ovariecotomized mice and was 2 fmol/mg in male mice. Administration of 17-β-estradiol increased PR content to 745 fmol/mg. In primary ovarian carcinomas, PR was significantly associated with ER concentrations (P < 0.0001), suggesting regulation of PR levels by estrogen. This association was present for tumors of endometrioid histology (P < 0.0001) but not for those with serous histology (P = 0.31). These data point to the regulation of PR levels by estrogen in ovarian cancer and to a mediatory role for PR in the inhibition of growth induced by progestin.

INTRODUCTION
The human PR2 is located at 11q22, and several observations suggest that it may have tumor-suppressing properties in ovarian cancer and mediate growth inhibition by progesterone in established tumors. A specific TaqI RFLP in the PR gene (1) has been associated with an increased frequency in ovarian cancer patients when compared with a control population (47 versus 22%; Ref. 2), although this requires confirmation (3). This polymorphism has been characterized as a 306-bp insertion into an intron (intron 0) of the gene and has been linked with mutations in exons 4 and 5, the latter leading to a change in amino acid from valine to leucine (4, 5). We have previously shown that loss of heterozygosity at the PR gene site is associated with reduced PR expression and poor survival in ER-positive ovarian cancers (6).

Indirect evidence of a role for PR in the growth regulation of established disease is provided by the association of PR with both survival and the responsiveness of some patients to progestins. A number of studies have noted a link between PR content in ovarian carcinomas and improved patient survival (7-11); however, this has not been a universal finding and may be dependent on the methodology and cutoff point used. Many small clinical studies have investigated the activity of progestins, predominantly megestrol acetate and medroxyprogesterone acetate, for the treatment of ovarian cancer, and although these have generally produced response rates of 10-20% (reviewed in Ref. 12), occasionally higher response rates of 45-53% have been reported (13, 14). Again, such data support the functionality of PR in at least some tumors, although the characteristics of this responding group have not been defined.

To further explore the endocrine regulation of ovarian cancer, we have developed model systems that encompass a range of PR and ER values (15-17). Using these systems, we have previously obtained evidence for the regulation of PR by estrogen in ER-positive ovarian carcinoma cell lines in vitro (17).

Here, we provide further evidence for a role of PR in this disease and the regulation of PR by estrogen in vivo. We have investigated the effect of the progestin megestrol acetate on the growth of a PR-positive ovarian cancer xenograft model and then determined whether PR levels can be regulated by estrogen in this system. The association between PR and ER in primary ovarian tumors was also investigated to determine whether PR levels were likely to be regulated via the ER in the tumors of patients.

MATERIALS AND METHODS

Xenograft Studies. Nude (nu/nu) mice (originally bred at Imperial Cancer Research Fund, London, United Kingdom) were obtained from Harlan OLAC and maintained in negative-pressure isolators. Mice were at least 8 weeks old at the time of experimentation. The PE04 ovarian carcinoma xenograft was initiated from the cell line by s.c. injection of 103 cells, and the HOX 60 ovarian carcinoma xenograft was initiated by implantation of fragments from the primary tumor into the flanks of female nude mice. These xenografts were then serially pas-
saged. Previously measured PR values for the PE04 and HOX 60 xenografts were 87 and 4 fmol/mg protein, respectively, whereas ER concentrations were 242 and 7 fmol/mg, respectively (16). For experiments in which the effects of hormonal modulation or castration were investigated, fragments of the xenografts were implanted s.c. into the flanks of animals. After ~1 month, when tumors had reached a mean volume of 32 mm³, animals were randomly allocated to treatment or control groups (each containing five to eight animals), and treatment was begun (defined as day 0). Megestrol acetate (1.5 mg/pellet; 60-day release) and 17β-estradiol (0.72 mg/pellet; 60-day release) were administered as slow-release pellets (Innovative Research of America, Sarasota, FL). These were implanted s.c. in the flank using a trocar. Ovariectomy of host nude mice was performed under anesthesia on day 0 in the castration experiment. Sera from non-tumor-bearing animals were collected in a separate experiment 7 days after ovariectomy or 17β-estradiol pellet implantation and analyzed as described below.

**Determination of ER and PR Concentration by Enzyme Immunoassay.** Tissue fragments (50–200 mg) were weighed and homogenized in buffer [10 mm Tris, 0.25 m sucrose, and 1 mm ethylenediamine tetracetae (pH 8.0) at 22°C, plus 1% mercapto-1,200 glycerol and 10% (v/v) glycerol, as described previously (18)]. After centrifugation at 105,000 × g, the supernatant cytosol was assayed by enzyme immunoassay using ER-EIA or PR-EIA kits (Abbott Ltd., Basingstoke, United Kingdom) according to the manufacturer’s instructions. The protein content of the cytosol was determined by the method of Bradford (19), and receptor concentrations were expressed as fmol/mg protein.

**Determination of Estrogen Concentration in Serum.** 17β-Estradiol was estimated by a RIA following diethyl ether extraction of serum (20). Intra- and interassay coefficients of variation averaged 12 and 13%, respectively, over the range 1 × 10⁻¹⁰–25 × 10⁻¹⁰ M. Assay sensitivity was 50 pmol/liter in a 100-μl sample volume.

**Patients and Tumor Samples.** Fresh primary ovarian tumor tissue was obtained from 82 patients with epithelial ovarian cancer, transferred to liquid nitrogen, and stored at −180°C until use. Tumor histology was assessed on paraffin-embedded sections and classified according to WHO criteria (21). Tumor histologies were classified as follows: serous adenocarcinoma (34 tumors), endometrioid adenocarcinoma (25 tumors), mucinous adenocarcinoma (8 tumors), clear cell carcinoma (5 tumors), malignant mixed mesodermal tumor (3 tumors), granulosa cell tumor (2 tumors), steroid cell tumor (1 tumor), Mullerian tumor (1 tumor), leiomyosarcoma (1 tumor), and undifferentiated carcinomas (2 tumors). The International Federation for Gynecology and Obstetrics staging, histopathology, and differentiation statuses were determined and reviewed in a standardized fashion at a multidisciplinary combined Gynecological Oncology Clinic. Data on stage of disease were available for 71 of these patients and were as follows: stage I, 24 patients; stage II, 3 patients; stage III, 37 patients; and stage IV, 7 patients. Data on survival were available for 69 patients. Treatment consisted of the best possible surgical debulking, followed by chemotherapy where appropriate.

**RESULTS**

**Progestin Activity against Ovarian Cancer Xenografts.** Megestrol acetate was given to groups of intact female mice bearing either the PR-positive PE04 or PR-negative HOX 60 ovarian carcinoma xenografts (Fig. 1). Significant growth inhibition of the PE04 xenograft was obtained after 7 days at a dose of 1.5 mg/mouse. This inhibition was maintained for ~10 days, but after this period, tumors regrew at a rate similar to that of controls, although the initial inhibition differential was maintained. In a subsequent experiment, lower (0.01 and 0.1 mg) and higher doses (10 and 50 mg) also produced statistically significant inhibition (~40% compared to controls) on day 10, but on day 23, only the lower doses still maintained inhibitory activity. Growth of the HOX 60 xenograft was unaffected at 1.5 mg/mouse, consistent with its background level (7 fmol/mg) of PR (Fig. 1).

**Estrogen Regulation of PR in an ER-positive/PR-positive Ovarian Carcinoma Xenograft.** To assess whether PR can be regulated by estrogen in ovarian carcinoma cells in vivo, the PR-positive PE04 xenograft was exposed to a number of endocrine environments. When grown in adult female mice, PE04 xenografts had a measured PR content of 145 fmol/mg...
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protein, and this fell to 7 fmol/mg in ovariectomized animals after 60 days. Implantation of 17-β-estradiol pellets into adult females raised PR content to 745 fmol/mg protein. When grown in adult male mice, the PR content of PE04 xenografts was measured as 2 fmol/mg (Fig. 2). These changes in PR values paralleled the level of circulating serum 17-β-estradiol (Fig. 2).

**PR Concentrations in Primary Ovarian Tumors and Relationships with Clinicopathological Parameters.** PR concentrations were measured in a series of 82 primary ovarian cancers. The median value for this group was 9 fmol/mg protein (range, 1-1211 fmol/mg protein), and the median value of PR in early-stage (I/II) tumors was significantly higher than that in advanced-stage (III/IV) tumors (12 versus 5 fmol/mg protein; $P = 0.007$ Mann-Whitney U test). This difference was irrespective of the histological subtype (Fig. 3). Endometrioid histology was associated with a higher median PR concentration (13 fmol/mg) than the other groups combined (median = 6 fmol/mg; $P = 0.009$, Mann-Whitney U test; Fig. 3). For the total group overall, there was no significant association between grade and PR content; however, in the endometrioid subgroup, PR concentration was higher in well-differentiated and moderately differentiated tumors than it was in their poorly differentiated counterparts (medians, 63 and 7 fmol/mg protein, respectively; $P = 0.03$; Fig. 4).

For PR, inspection of the data indicated that a higher tumor content of steroid receptor was associated with improved survival. Selection of the arbitrary value of 40 fmol/mg protein produced a highly significant association ($P = 0.0007$) between tumor concentration and survival, whereas subdivision into <5 fmol/mg, 5-40 fmol/mg, and >40 fmol/mg produced a $\chi^2$ for trend of 0.002 (Fig. 5).

**Relationship of ER and PR.** For the malignant group overall, there was a highly significant relationship between ER and PR concentration ($P < 0.0001$; Fig. 6). Analysis of the data separately for each histological subtype indicated that this relationship was particularly strong in the endometrioid group ($P < 0.0001$) but absent in the serous group ($P = 0.31$; Fig. 7). The numbers of mucinous and clear cell tumors were too few to draw firm conclusions.

**DISCUSSION**

The importance of steroid hormone receptors in ovarian carcinoma, in particular their role in growth regulation of established disease, and the value of hormonal manipulations for the treatment of ovarian cancer are, at present, poorly defined. Both progestins and antiestrogens have produced responses in a percentage of ovarian cancer patients, but response rates between clinical trials vary enormously. Although several clinical trials with progestins have produced impressive response rates of up to 50% (13, 14), most trials have produced response rates on the order of 10-20%, and some have failed to detect any (reviewed in Ref. 12). For example, use of high-dose megestrol acetate in three consecutive ovarian cancer trials produced response rates of 43% (10 responses in 23 patients; Ref. 13), 8% (4 responses in 47 patients; Ref. 22), and 0% (0 responses in 35 patients; Ref. 12). The reasons for these variations are unclear; however, given the low toxicity profiles and potential use of these agents in chemoresistant disease, it would be desirable to identify features that predict sensitivity. To address this question further, we examined the effect of the progestin megestrol acetate in two xenograft models. The PR-positive xenograft...
responded to megestrol acetate whereas the PR-negative HOX 60 was unresponsive, and this was consistent with its lack of receptors. The duration of the response in the PEO4 xenograft was limited, and this may be related to down-regulation of PR by continuous exposure to progestin. Such an effect has been demonstrated in a PR-positive endometrial carcinoma grown as a xenograft model (23, 24). This might also explain why higher doses (10 and 50 mg/pellet) had a decreased effect after several weeks compared to lower doses. These results suggest that the presence of receptors is required for response but that continuous exposure to progestin may lead to only a limited growth inhibition.

Previous studies of PR expression and their associations with clinical and pathological features of ovarian cancer have yielded widely varying results, possibly dependent on the methodology and limited quantitative assessments in many of these studies (reviewed in Ref. 25). Here, using enzyme immunoassay, we were able to confirm several of the most significant observations obtained previously using dextran-coated charcoal methodology. We found a higher median level of expression in early stages of the disease, which has also been detected by Harding et al. (10). The observation of a greater median expression in the endometrioid subtype has also been observed by several groups, but again, this is not a universal finding (25). The most interesting link, however, is the finding that increased PR is found in tumors that are associated with a better outcome; this indirectly suggests a possible role in the regulation of growth. The previous positive findings had used cutoff points of 9–10 fmol PR/mg protein (7, 9, 11), 20 fmol PR/mg protein (10), and 50 fmol PR/mg protein (8). Our finding of 40 fmol PR/mg protein proving to be a useful cutoff point is in agreement with the last mentioned of these studies, suggesting that a moderate to high level of PR is related to good prognosis.

Previous in vitro experiments had demonstrated an induction...
Fig. 5 Relationship between PR content and survival of patients with ovarian cancer. PR was measured by enzyme immunoassay, as described in "Materials and Methods." Kaplan-Meier curves were compared. $P = 0.002$, $\chi^2$ for trend.

Fig. 6 Relationship between PR and ER in primary ovarian cancers. Both PR and ER were measured by enzyme immunoassay, as described in "Materials and Methods." PR and ER were compared by the Spearman rank correlation ($P < 0.0001$).

of PR after 17-β-estradiol treatment in the ER-positive PEO4 carcinoma cell line (17). Here, in vivo reduction of circulating levels of estrogen by ovariectomy reduced PR concentration in the PEO4 xenograft, whereas exogenous administration increased PR concentration, supporting the view that PR levels are estrogen regulated. Consistent with this result, the PR concentration in PEO4 xenografts grown in adult male mice was at the limit of receptor assay sensitivity against a background of low estrogen levels. Hamilton et al. (26) showed increased progestin binding in the OVCAR-3 xenograft after exogenous estrogen. Together, these data strongly support the view that 17-β-estradiol can regulate PR in ER-positive ovarian cancer cells.
If estrogen regulation occurs in the tumors from patients, it would be expected that PR concentrations would be increased in tumors with a higher ER content, so the association between PR and ER in a series of primary ovarian tumors was explored. Overall, there was a good correlation between these two parameters, implying that regulation of PR may be under estrogen control in this disease, an association in agreement with the experimental models. However, analysis of individual tumor subtypes revealed that the association among different forms of ovarian cancer was not uniform, and although the correlation in the endometrioid group was very strong, it was absent in the serous group. The absence of an association in the serous group may be at least partially explained by common losses on chromosome 11q22, at or close to the PR gene, which, in a previous study, had been demonstrated to be found in 7 of 15 serous tumors but in 0 of 9 endometrioid tumors (6). The difference between endometrioid and serous groups may also be linked with possible alternative origins of the disease. Both endometrioid and clear cell ovarian carcinomas have been associated with endometriosis, a condition in which tissue histologically similar to that of the endometrium is found at sites outside the uterine cavity, most commonly the ovary (27, 28). Although endometriosis is generally benign, it has been found at much higher rates in women with endometrioid ovarian cancer (26%) than in women with serous or mucinous tumors (3–6%; Ref. 29); furthermore, malignant transformation has been documented (28).

It is feasible, therefore, that endometrioid and clear cell carcinomas may arise through malignant transformation of endometriotic lesions (27, 28). Because ER and PR are tightly regulated in endometrial tissue, it would be reasonable to speculate that the same might be true in ovarian endometrioid tumors if these were derived from the same tissue.

In a previous study, we reported that patients with ER-positive/PR-negative tumors had poorer survival than those with ER-positive/PR-positive ovarian cancer (6). It is feasible that estrogen, via the ER, drives both growth-stimulatory pathways, e.g., induction of mitogenic growth factors such as transforming growth factor-α (30), and growth-inhibitory pathways such as PR. If the PR acts as a growth-inhibitory route as the above data suggests, then, if present, tumors should have a better outcome if PR is functional and sufficient circulating progesterins are available to act. This could then explain the differential in survival between the above two groups of patients.

In conclusion, these data provide further support for the view that PR is both functional and under estrogen control in ovarian cancer.

REFERENCES


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