10-28-2003

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Reproductive Biology and Endocrinology

BioMed Central

Review

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Published: 07 October 2003

Reproductive Biology and Endocrinology 2003, **1**:73

Received: 24 July 2003
Accepted: 07 October 2003

This article is available from: http://www.RBEj.com/content/1/1/73

Abstract

Ovarian carcinoma (OCa) continues to be the leading cause of death due to gynecologic malignancies and the vast majority of OCa is derived from the ovarian surface epithelium (OSE) and its cystic derivatives. Epidemiological evidence strongly suggests that steroid hormones, primarily estrogens and progesterone, are implicated in ovarian carcinogenesis. However, it has proved difficult to fully understand their mechanisms of action on the tumorigenic process. New convincing data have indicated that estrogens favor neoplastic transformation of the OSE while progesterone offers protection against OCa development. Specifically, estrogens, particularly those present in ovulatory follicles, are both genotoxic and mitogenic to OSE cells. In contrast, pregnancy-equivalent levels progesterone are highly effective as apoptosis inducers for OSE and OCa cells. In this regard, high-dose progestin may exert an exfoliation effect and rid an aged OSE of pre-malignant cells. A limited number of clinical studies has demonstrated efficacies of antiestrogens, aromatase inhibitors, and progestins alone or in combination with chemotherapeutic drugs in the treatment of OCa. As a result of increased life expectancy in most countries, the number of women taking hormone replacement therapies (HRT) continues to grow. Thus, knowledge of the mechanism of action of steroid hormones on the OSE and OCa is of paramount significance to HRT risk assessment and to the development of novel therapies for the prevention and treatment of OCa.

Introduction

Ovarian carcinoma (OCa) continues to be the leading cause of death due to gynecologic malignancies. Over 23,300 will be diagnosed and 13,900 patients will die in 2003 from OCa [1]. The incidence of OCa varies widely in frequency among different geographic regions and ethnic groups, with high incidences observed in Scandinavia, Western Europe and North America and low incidences found in Asian countries [2]. The majority of cases is sporadic while about 5% to 10% of OCa is familial. Although all cell types of the human ovary may undergo neoplastic transformation, the vast majority (80–90%) of benign and malignant tumors are derived from the ovarian surface epithelium (OSE) and its cystic derivatives [3,4]. The origin of OSE could be traced to the mesothelium of the embryonic gonads, or the Mullerian epithelium, and therefore OCas often resemble those of the fallopian tube, endometrium, and endocervix [4,5].

Incessant ovulation is a probable cause of ovarian carcinogenesis

A long-standing hypothesis has been proposed to explain the causal mechanism of ovarian carcinogenesis. The "incessant ovulation" hypothesis argues that repeated cycles of ovulation-induced trauma and repair of the OSE at the site of ovulation, without pregnancy-induced rest periods, contributes to ovarian cancer development [6,7]. According to this hypothesis, successive bouts of apopto-

sis and regenerative repair of OSE cells at the ovulation site induces genetic instability, which predisposes this cell layer to tumorigenesis. Studies in the ewe provided experimental evidence in support of this theory. Oxidative DNA damage, expression of p53, and apoptosis occur among the OSE cells within the formative site of ovulation [8]. Concomitantly, the anti-apoptotic Bcl-2 and the base excision repair enzyme polymerase-beta are overexpressed in OSE cells at the margins of ruptured follicles. Following exfoliation of OSE cells at the dome of the ovulatory follicles, repair is accomplished by replication of cells at the margin and their migration to the wound [9]. Survival and subsequent clonal expansion of OSE cells with unrepaired genomic damage and heightened survival potential may present itself as a risk factor for ovarian carcinogenesis. Therefore, with each cycle of ovulation an increased number of genetically altered cells accumulates in the OSE and raises the risk of tumor initiation. In agreement with findings in the sheep, a recent study reported that tumorigenecity in human OCa cells collected from effusions was associated with abnormal expression of p53, which plays a key role in regulating DNA damage-induced apoptosis [10].

Hormone replacement therapy (HRT) and ovarian cancer risk

As life expectancy increases in most countries, progressively more women are expected to spend over one third of their life post-menopausally. To ameliorate symptoms of the climacteric, primarily vasomotor flashes and sweats, estrogen-based hormone replacement therapy (HRT) is used by millions of women around the world. For some, longer-term usage (> 5 years) is often necessary to relieve persistent symptoms. Others continue to use the therapy for prevention of a number of aging-related diseases including osteroporosis, myocardial infarction, strokes, dementia, and possibly Alzheimer's disease. Justifications for longer-term usage are in part based on findings from observational studies that suggest a protective effect of estrogen against bone loss, atherosclerosis and memory deficit [11]. However, recently published results from a randomized trial, the Women's Health Initiative (WHI), indicate significant increases in the incidences of breast cancer, thromboembolic diseases, and dementia in women using the HRT with combined estrogen and progestin [12,13]. These results have led to the early termination of this arm of the trial and raised great concerns among women and the medical community regarding the safety of HRT usage. The premature termination of the estrogen plus progestin arm makes it difficult to interpret and compare results from other arms of the WHI trial and has significantly undermined future trials that aim at testing the impacts of other post-menopausal therapies on disease development, including OCa.

Observational data suggest there may be a small increased risk of OCa associated with longer-term use of HRT [14–16]. However, the evidence from these studies is in disagreement with other reports that detected an unchanged [17,18] or a reduced [19,7,20] risk of developing the cancer. Intriguingly, the Heart and Estrogen/progestin Replacement Study (HERS) II trial, a randomized trial of post-menopausal hormones, has recently reported no difference in OCa incidence between the HRT and the placebo group [21]. Since placebo controlled, randomized clinical trials are usually considered to be "gold standards" to assess the real risks and benefits of chronic treatments, results from the HERS II might ease concerns regarding HRT and OCa risk among some users. Explanations for the discrepancies among various studies and those between observational studies and clinical trials have been attributed to differences in methodologies and existence of uncontrolled confounding factors. To date, questions pertaining to the effects of estrogens and/or progestins on ovarian carcinogenesis remain unanswered.

Estrogens are implicated as causative factors of ovarian carcinogenesis

Estrogens have long been suspected as etiologic factors of OCa. Although usage of estrogen-based oral contraceptives is known to reduce OCa risk, its effect is primarily attributed to reduction in ovulation frequency. Ovarian tissue estrogen levels are at least 100-fold higher than circulating levels and those in the follicular fluid of ovulatory follicles are even higher [22]. Thus, OSE and its cystic derivatives are likely to be exposed to high levels of these steroids. Literature from breast cancer research has demonstrated direct genotoxic effects of estrogen [23]. Hence, it is logical to speculate that genomic damage of OSE cells, covering the ovulating follicles or in inclusion cysts may, in part, be caused by the high levels of estrogen in the follicular fluid or in the ovarian stroma.

In addition to inducing genetic damages to OSE cells, Syed et al [24] have reported estrogen receptor (ER)-mediated growth stimulatory responses of normal and malignant OSE cells to estradiol-17 beta and estrone. Worthy of mentioning is the observation that estradiol-17 beta and estrone are of equal potency in stimulating growth in OSE cells although it is well known that estrone is a much less potent estrogen when compared to estradiol-17beta. This is an important finding because, after menopause, estrone is the major circulating estrogen produced as a result of aromatization from androstenedione in skin and adipose tissue [25]. In this regard, a recent prospective study on postmenopausal women found higher OCa mortality rates among overweight [Body Mass Index (BMI) >/ = 25] and obese (BMI >30) women [26] suggesting that peripheral estrogen formation is probably a promotional factor for OCa progression. Furthermore, it was demonstrated

that the mitogenic effects of estrogens, and those of androgens and gonadotropins, on OSE cells were mediated through activation of the IL-6/STAT-3 signaling pathway and that OCa cells expressed high levels of constitutively activated STAT-3, a known transforming cellular molecule [27]. Lastly, a recent study examined primary cell cultures derived from twenty-five OCa patients and found that tumor cells secreted estradiol-17 beta, but not testosterone nor progesterone. In this study, the estrogen was shown to exert antiapoptotic effects on OCa cells. The ability of OCa cells to produce estradiol-17 beta implicates an auto-/paracrine-influence of this steroid on OCa progression.

Taken together, it is reasonable to speculate that the combined genotoxic and mitogenic effects of estrogens constitute a potent force for the neoplastic transformation of normal OSE cells. In addition, circulating and/or *in situ* produced estrogens, via growth stimulation and inhibition of apoptosis, likely play critical roles in tumor initiation and promotion [28,24].

Progesterone is implicated as a protective factor against OCa

Progesterone (P4) or cellular responses to P4 appears to offer protection against ovarian carcinogenesis. Of significance, loss of heterozygosity at 11q23.3-24.3 that harbors the progesterone receptor (PR) gene locus is commonly observed in OCa specimens [≈75%, [29–31]] and this genetic alteration is associated with poor prognosis [31]. These findings thus implicate PR as a tumor suppressor gene. Epidemiological data provide additional support that P4 or response of OSE cells to the steroid affords a protective role against OCa development or progression. An increase in ovarian cancer incidence was observed among women with progesterone deficiency [32]. In contrast, increased parity is associated with a reduction in OCa risk [33,34]. The protective effect of pregnancy may be attributable to exposure of the OSE to high levels of P4 during pregnancy [35]. In concordance, women with a history of twin pregnancies exhibit a lower risk for developing OCa, possibly due to higher levels of P4 found in maternal circulation during twin pregnancies when compared to singleton pregnancies [36,33]. Intriguingly, unlike breast cancer, a pregnancy at advanced age is more protective than one at young age against OCa [34]. This later observation supports the "exfoliate" theory of OCa protection. It theorizes that pregnancy levels of P4 rid the OSE of genetically damaged, pre-malignant cells and hence reduce the risk of tumorigenesis during subsequent years.

Expression of progesterone and estrogen receptors in OSE and OCa

Until recently, little was known about expression levels of the estrogen receptors (ERs) and PRs in ovarian tumors or in normal OSE. Using semiquantitative RT-PCR, Lau and co-workers [37] demonstrated expression of ER-alpha and ER-beta mRNA, as well PR mRNA, in primary cultures of normal OSE cells. A moderate reduction in ER-alpha mRNA expression, accompanied by a marked downregulation of PR expression, was noted in OCa cell lines when compared OSE cells. Lee et al [38] reported 86% of ovarian tumor specimens stained positive for ER, 50% positive for PR, and 45% positive for both receptors. In another study, PR immunopositivity was observed in the majority of borderline tumors, whereas almost all (93%) malignant ovarian tumors stained negative for PR [39]. Among the various OCa subtypes, ER-alpha immunostaining was found in 97% of serous adenocarcinomas, 100% of endometrioid adenocarcinomas, 70% of mucinous adenocarcinomas and none of the clear cell carcinoma specimens (0%) [40]. In contrast, ER-beta immunopositivity was found in all OCa subtypes (39% of clear cell carcinoma, 41% of serous adenocarcinoma, 30% of mucinous adenocarcinomas, and 75% of endometrioid adenocarcinoma). Except for clear cell carcinomas, PR was expressed in 30% to 70% of the various OCa subtypes in this study. Another investigation had compared expression levels of the two major PR isoforms, PR-A and PR-B, in ovarian tumors and in normal and benign ovarian tissues [41]. No significant difference was noted in the expression levels of PR-B among normal/benign ovarian tissues and cancerous specimens. In contrast, PR-A was found to be expressed in normal and benign ovarian tissues but exhibited marked reduction in malignant cancer specimens. In ovarian cancer cell lines (OVCAR-3 and Caov-3), the PR-B/PR-AB mRNA ratio was elevated by estradiol-17 beta in both a time- and dose-dependent manner [41]. However, this ratio was unaltered when a normal OSE cell line (NOV-31) was exposed to the steroid. At the protein level, treatment with estradiol-17 beta markedly upregulated PR-B expression in OVCAR-3 while both PR-A and PR-B isoforms were elevated in NOV-31 by the hormone treatment. All in all, it appears that the two PR subtypes are differentially regulated by estrogen and differentially expressed between normal OSE and OCa. A loss of PR-A was found to be associated with ovarian malignancy. The cause for the loss of PR expression is unknown but may be related to a diminution in estrogen responsiveness in OCa cells [40] and/or a second 'hit' following loss of PR heterozygosity [29–31]. In this regard, somatic mutations in the primary sequences of PRs have not been reported in OCa. Since a recent study has demonstrated DNA hypermethylation-mediated silencing of PR-B in human uterine endometrial cancer [42] this mechanism of transcrip-

tional inactivation of PRs in OCa needs to be investigated in future studies.

PROGINS refers to a group of complex PR gene polymorphisms [43,44]. It includes a polymorphism in intron G of the human PR, caused by an Alu insertion, a G to T substitution in exon 4, causing a Valine to Leucine change in the hinge region of the receptor, and a synonymous C to T substitution in exon 5 reported to be linked to the Alu insertion. It was demonstrated that the PROGINS allele codes for a PR with increased stability and increased hormone-induced transcriptional activity. PROGINS polymorphism was reported to be associated with OCa in a number European and North American Caucasian populations [43,45,46] and the PROGINS allele has been speculated as a modifying gene in hereditary OCa. However, a recent study in an Australian population found no association between PROGINS and increased risk for OCa [44]. Additionally, the protein levels of ER and PR in OCa specimens were independent of the PROGINS status [44].

Some studies did not find a strong correlation between OCa progression and PR and/or ER gene expression [47,41]. However, one recent report has demonstrated that PR-B labeling index (immunopositivity) is an independent prognostic factor for OCa [48]. In addition, it was shown that the ER-negative and PR-positive (ER-PR+) OCas, which accounted for approximately 10% of all tumors, showed a significantly superior prognosis when compared with all other combinations of ER and PR expression statuses [49]. Five-year survival rate was over 80% for ER-PR+ tumors versus 45% for tumors expressing all other steroid hormone receptor combinations. Finally, it has been speculated that the loss of ER expression in OCa may explain the disappointing responses of OCa patients to antiestrogen therapy such as tamoxifen [50].

Mechanism of P4-mediated anti-OCa action

A handful of *in vitro* studies have demonstrated an inhibitory action of P4 on OCa cell growth [51–54,27]. Others showed a clear induction of apoptosis in OCa cells by the steroid [51–54]. Alterations in apoptosis regulators such as bcl-2, c-myc and p53 were detected in OCa cells undergoing apoptosis [52,30]. Relatively high doses of P4 (1 μM range) were used in these studies to achieve growth inhibition and apoptosis. In a recent investigation, the effects of P4 on normal OSE and OCa cells were studied simultaneously across a 6-log concentration range (10^{-11} to 10^{-6} M). Results from this study demonstrated a growth promoting effect of P4 at low concentrations (below 10^{-8} M) and a growth inhibitory action of P4 at higher concentrations [24]. These observations are consistent with the belief that regenerative proliferation of the OSE after ovulation involves P4. Furthermore, P4 may promote repair of ovulation-induced genomic damages during early luteal phase via induction of polymerase-beta activity as suggested by experiments in OSE cells derived from the ewe [9]. In contrast, higher doses of P4, in the range experienced during pregnancy or perhaps during oral contraceptive usage, induce cell cycle arrest or apoptosis in normal OSE cells and OCa cells. Of significance, Syed and Ho [55] recently demonstrated that the P4-induced apoptosis in both normal OSE and OCa cell cultures utilized an extrinsic apoptosis-initiation pathway involving activation of caspase-8 rather than the intrinsic, mitochondrion-related caspase-9 pathway. They further showed that the P4-induced caspase-8 activation led to an enhancement of Fas/FasL signalling which might ultimately be responsible for apoptosis induction in OSE and OCa cells. In concordance, an earlier study found that the cytotoxic effect of an anti-Fas antibody on an OCa cell line (NOS4) was dependent on the activation of caspase-8 [56]. Among the TNF receptor family, Fas (APO-1/CD95), is recognized as an important death receptor that serves to transmit extracellular apoptotic signals intracellularly [57,58]. Activation of the receptor is normally accomplished by binding of the Fas ligand (FasL) but could also be achieved by interaction with an anti-Fas antibody [59,60]. The Fas/FasL system was first known to play an important role in eliminating self-damaging T cells. More recently, this signalling system has been shown to be a prime mediator of therapeutic cell kill in a variety of cancer cells, including those derived from the OSE [61]. In conclusion, these studies now establish a connection between P4 action and Fas/Fas L signaling in normal and malignant OSE cell death.

Another mechanism by which P4 exerts its antitumorigenic action is via reduction of fluid dynamics of plasma membranes in OCa cells [62]. The decrease in membrane fluidity has been demonstrated to be related to *in vitro* inhibition of exocytotic vesicle release, cell invasiveness in Matrigel, and colony formation in collagen matrix. Importantly, inhibition of *in vivo* tumorigenesis in immunocompromised nude mice has also been demonstrated in this study.

Lastly, new evidence has emerged that indicates the antitumorigenic effects of P4 may be mediated by induction of alternative expression of transforming growth factor-beta (TGF-beta) isoforms in OSE. When ovaries from the control and estrogen-only-treated monkeys were compared to the ovaries of progestin-treated monkeys a marked decrease in the expression of TGF-beta1 and a concomitant increase in the expression of the TGF-beta2/3 isoforms was observed in the OSE. The P4 induced switch from TGF-beta1 to TGF-beta 2/3 expression was highly correlative to increased apoptotic index in the OSE.

Clinical use of antiestrogens in the treatment of OCa

Initial clinical studies demonstrated that the use of tamoxifen, a first generation antiestrogen, for relapsed OCa treatment was far from effective [63,64]. In one study, when 105 patients with Stage III or IV epithelial OCa with recurred disease were treated with tamoxifen 10% demonstrated a complete response, 8% showed a partial response, and 38% had short-term disease stabilization [63]. The response or stabilization period was between 7 to 19 months and patients with high levels of ER appeared to respond better. Another study has used the antiestrogen in cisplatin-refractory OCa patients and demonstrated a modest objective response rate of 13% [65]. Therefore, the use of tamoxifen alone for treatment of OCa has made little advancement since these earlier trials. Recently, it has been shown that tamoxifen may be able to synergize standard platinum-based OCa treatment [66]. Fifty patients with recurrent OCa received platinum-based chemotherapy (cisplatin or carboplatin) before they were treated with tamoxifen. This combined treatment regimen was found to produce an overall response rate of 50% (complete response 30%; partial response 20%) with a median duration of 8.5 months (3–42 months). These results have raised optimism that an antiestrogen could be used in combination with platinum-based therapies to provide more effective treatments for OCa. Furthermore, with the new knowledge that OCa cells may produce their own estrogens [25] the use of an aromatase inhibitor may further enhance the response. In a small clinical study, 50 patients were treated with the aromatase inhibitor letrozole and evaluated by UICC criteria [67]. Although no complete or partial responses were obtained, 10 patients had stable disease on scan for at least 12 weeks. Letrozole treatment also induced higher levels of ER expression in the tumors, a condition believed to favor a response to antiestrogen. Findings from these trials suggest that treatment of patients with letrozole alone or in combination with other therapeutic regimens may result in longer-term survival in subgroups of patients.

Clinical use of progestins as chemopreventive and treatment agents against OCa

Several pre-clinical studies have tested the efficacy of P4 to prevent OCas. Using the laying hen, *Gallus domesticus*, as a model of spontaneous ovarian carcinogenesis, it was found that medroxyprogesterone acetate (MPA or Depo-Provera) reduced the frequency of spontaneously developing reproductive tract adenocarcinoma including OCa in the avian model [68]. The mechanism of action of P4 is believed to be directly related to a reduction of ovulatory events in this avian species. Similarly, it has been demonstrated that monkeys treated with the progestin-component of the oral contraceptive (levonorgestrel) have increased apoptosis in the ovarian epithelium cells as compared with controls and ethinyl estradiol-treated monkeys [69]. Thus results from the latter study suggest that the anti-OCa effects of P4 may be beyond inhibition of ovulation but more directly related to its pro-apoptotic action on OSE cells. As discussed above, removal of OSE cells reduces the number of cells with pre-malignant lesions in this tissue, which has the propensity to accumulate genetically damaged cells through repeated ovulations.

Injectable progestins have been widely used as contraceptives by women of the third world under the USAID program [70]. Depot medroxyprogesterone acetate (MPA), marketed under the brand name Depo-Provera, taken every 3 months, is by far the most commonly used [71]. Another progestin-only injectable is norethindrone enanthate (NET EN), taken every 2 months. It is estimated that more than 30 million women in 90 countries have used injectable progestins to prevent conception with a current usage rate of 9 million, most popular among women in Thailand and Indonesia. In 1992, the US Food and Drug Administration (FDA) approved the use of Depo-Provera as a contraceptive in the United States. Furthermore, Depo-Provera has been approved for non-contraceptives usage such as treatment of endometrial cancer since 1969. However, progestins have not been considered as a chemopreventive agent for high risk populations primarily due to concerns of a possible link between long term progestin usage and risks of breast and cervical cancers [14,72]. Recent research, however, demonstrated no connection between Depo-Provera usage and cervical and ovarian cancer risk. The progestin was further shown to reduce endometrial cancer incidence [70]. Moreover, a recent observational study found that survival in 33 patients with poorly differentiated OCas correlated with higher serum progesterone, especially in combination with expression of PR [73].

The major unease that prevents considering using progestins as chemopreventive agents for OCa resides in the possibility of increased breast cancer risk. Based on the weight of current experimental and observational study evidence a rationale approach would be to treat high risk women with pregnancy equivalent level of progestin for a short duration in order to induce the "exfoliation" effect of P4 on the OSE. Future clinical trials are needed to determine the efficacy of high dose progestin treatment in OCa prevention.

An earlier randomized trial demonstrated that MPA or MPA plus tamoxifen were not effective in causing OCa regression. However, both treatments stabilized the cancer for 4 to 16 months [74]. Data from a small clinical study on stage III recurrent OCa patients suggested that

progesterone combined with platinum-based chemotherapy as a first-line therapy may improve the prognosis of advanced epithelial ovarian cancer, but had little effects on the prognosis of early stage epithelial OCa [75].

Conclusion

Despite recent gains in our knowledge of regulation of cell proliferation and apoptosis in normal OSE and in OCa by estrogens and progesterone, the relationships between these steroids and ovarian carcinogenesis remain poorly understood. The weight of evidence suggests that estrogens, particularly those present in the intra-follicular fluid of ovulatory follicles, may contribute to initiation and/or promotion of ovarian carcinogenesis, whereas progesterone, at levels equivalent to those found during pregnancy, may offer protection against OCa development. The current knowledge base also reveals significant differences exist in the responses of various female tissues to estrogen and progesterone, or their combined action. Although the molecular bases of these differences are not fully understood it is safe to speculate that they are mediated, in part, by the different levels of ER and PR subtypes expressed in these tissues. Additionally, expression levels of the various steroid receptor co-regulators must also play an important role in modulating tissue sensitivity and responsiveness. From a clinical perspective, a more in-depth understanding of the action of estrogens and progestins on normal and malignant OSE cells is of paramount significance to HRT risk assessment. Furthermore, if issues revolving around the adverse effects of progestins on mammary carcinogenesis could be resolved, the use of progestins for OCa prevention in high risk populations would become an attractive option. As selective estrogen receptor modulators have been developed to circumvent the uterine cancer risk of estrogen-based HRT, selective progesterone receptor modulators could be produced in the future to offer protection against OCa while sparing the mammary gland of a tumor promoting action. Finally, the combined use of progestins with various chemotherapeutic agents continues to hold promise for new treatment regimens to be developed for OCa.

Acknowledgements

Research in part supported by a NIH-NCI grant #CA94221, a US Army Ovarian Cancer Research Program Ideal Award #DAMD17-99-1-9563 and a Mary Kay Ash Foundation Research Award #MKACF02-01 awarded to SMH.

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