Expression of Gonadotropin and Activin Receptor Messenger Ribonucleic Acid in Human Ovarian Epithelial Neoplasms

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ABSTRACT
Activin receptors (ActRs) and gonadotropin receptor mRNA expression were investigated in 18 human ovarian epithelial neoplasms. Northern blot analysis showed the presence of 3.0-kb type Ia ActR, 6.0- and 3.0-kb type Ila ActR, and 5.0-kb type Iib ActR mRNA transcripts in total RNA prepared from the cancer tissues. One carcinoma showed two major transcripts of a follicle-stimulating hormone receptor (FSH-R) gene, 4.1 and 2.4 kb, whereas the other two carcinomas showed two major transcripts of the luteinizing hormone/human chorionic gonadotropin receptor (LH-R) gene, 5.4 and 2.4 kb. These results were further analyzed by studying the corresponding PCR-amplified FSH and LH-R cDNA obtained by reverse transcription of total RNA. Expression of FSH-R mRNA was confirmed in about half of the cancer tissues. The size of the FSH-R reverse transcription-PCR product was the same as in normal ovarian follicles. Similarly, expression of LH-R mRNA was also detected in about half of the cancers.

Normal ovaries and cancer tissues were homogenized, and activin concentrations were measured in extracts. Activin levels in normal ovarian tissues were around 0.59 ± 0.01 ng/mg protein (mean ± SE; n = 5), and activin production was detected in every cancer tissue, except one—serous adenocarcinoma. The findings in this study demonstrated that activin and ActRs are present in and synthesized by human ovarian epithelial neoplasms. Thus, activin seems to be available as an autocrine/paracrine factor in epithelial neoplasms and may contribute to the expression of FSH-R, although the roles of activin and gonadotropin in tumorigenesis has yet to be defined.

INTRODUCTION
Ovarian epithelial cancer is the most common malignant ovarian neoplasm and a leading cause of death from gynecological malignancies in women. The factors that regulate the rapid growth of ovarian epithelial carcinoma and other types of malignant tumors are still largely unknown. Recently, the search for the molecular and cellular basis of malignant transformation has resulted in the integration of studies of oncogenes and peptide growth factors. It has been suggested that the autonomous growth of transformed cells might be due to the constitutive expression of growth factors and membrane receptors or a lack of response to growth-restricting signals (1–3). In support of this concept, many types of tumor cells have been found to release polypeptide growth factors when they grow in culture, and the same tumor cells often possess functional receptors for the peptides released (4). These factors acting in an endocrine, paracrine, and/or autocrine manner may stimulate or inhibit the proliferation of different cells. In recent years, techniques have been developed to culture normal human ovarian epithelial cells and malignant cells from ovarian cancer patients (5). These studies have shown that ovarian surface epithelial cells retain sensitivity to a number of growth factors and may require their presence for proliferation.

Activin, inhibin, and follistatin are protein hormones, all of which were originally isolated from gonads as regulatory factors of pituitary FSH secretion. Inhibin and activin are structurally related glycoproteins, consisting of two subunits linked by disulfide bonds (6–9). Inhibin is composed of a subunit and one of the two β subunits (βα or βb), whereas activin is formed from a combination of two of the same or different β subunits. Because the actions of activin are mediated by binding to and/or activating type I and II serine/threonine kinase receptors (10), the expression of these receptors is required for a cell to respond to activin. Elevated levels of inhibin and its subunits have been detected in subjects with a variety of gonadal stromal tumors (11–13), most commonly granulosa cell tumors (14–16). A recent study of human epithelial ovarian cancer cell lines revealed the presence of the activin type II receptor and βA and/or βB subunit expression and secretion (17). In addition, activin A seems to be available as an autocrine/paracrine factor in epithelial ovarian tumors and may contribute to circulating levels, but its role in tumorigenesis has yet to be defined (18).

LH and FSH are heterodimeric glycoprotein hormones synthesized and secreted by the pituitary, and both hormones bind to distinct receptors in the gonads and regulate several
aspects of gonadal growth, differentiation, steroidogenesis, and ovulation. Several epidemiological studies have indicated a direct correlation between the number of ovulations during reproductive life and the incidence of surface epithelial cancers (19–21). Use of oral contraceptives and high parity seems to have a protective effect, presumably by reducing the number of ovulations. Several other studies have suggested that the gonadotropins play important roles as trophic factors for gonadal tumor development. Gonadotropins are important cell survival factors in the testis of male rats, and targeted overexpression of a LH analogue in transgenic mice leads to ovarian tumors (22, 23). In elderly women, elevated FSH levels are associated with the development of ovarian cancers (24). These observations raise the possibility that gonadotropins may be linked to the development of ovarian cancer; the expression status of gonadotropin receptor in these tumors is, thus, of considerable interest. In the present study, we have examined whether epithelial ovarian tumors express activin and ActRs and whether activin production has some relation to the expression of FSH-R in ovarian epithelial cancer.

MATERIALS AND METHODS

Patients. The subjects were seen at Gunma University Hospital between September 1993 and December 1997. Normal ovaries were removed from patients who had undergone salpingo oophorectomy for gynecological disease. Pathological examination of the ovaries revealed no apparent abnormalities, and none of the patients had received hormone treatment for 6 months prior to surgery. A total of 18 women with ovarian carcinoma underwent primary surgical treatment. The project was approved by the committee on Investigation Involving Human subject of Gunma University School of Medicine. Tumor tissue and normal ovaries were obtained at the time of surgery and were immediately frozen in liquid nitrogen. They were stored at −80°C prior to use. Informed consent was obtained from the patients. A portion of each specimen was fixed with 10% formalin and embedded in paraffin. For routine histological studies, paraffin sections were stained with H&E, and all sampled areas were examined histologically. The ovarian epithelial tumors were histologically typed according to the classification system of the WHO.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from the isolated homogeneous tissue or cultured cells using the guanidium thiocyanate method (25). The final RNA pellet was dissolved in dimethyl pyrocarbonate-treated water, and total RNA was quantified by measuring the absorbance of the samples at 260 nm. For Northern blot analysis, 10 μg of total RNA from each tissue was separated by electrophoresis on denaturing agarose gels and subsequently transferred to a nylon membrane (Biodyne, ICN). Northern blots were hybridized at 68°C with digoxigenin-labeled cRNA probes. In accordance with the standard protocol for the nucleic acid detection kit used (Boehringer Mannheim), membranes were then exposed to Kodak X-0mat film (Eastman Kodak, Rochester, NY). Human type I ActR (ActRI) cDNA and rat type IIb ActR (ActRIIB) were subcloned into the Bluescript vector and linearized using SacI and HindIII, respectively. Rat type IIA ActR (ActRIIA) was subcloned into the Bluescript vector and linearized using BamHI. Human FSH-R and LH-R cDNA (26, 27) were subcloned into the EcoRI site of the Bluescript vector and linearized using HindIII and BamHI, respectively. Digoxigenin-labeled cRNA probes were produced by in vitro transcription using T7 or T3 RNA polymerase and using an RNA labeling kit (Boehringer Mannheim). Digoxigenin-labeled β-actin cRNA probes were obtained using the same method.

RT-PCR Analysis. For PCR amplification, 3 μg of poly(A)+ RNA was used to generate first-strand cDNA using a cDNA synthesis kit (Life Technologies, Inc.), following the manufacturer’s instructions. The entire 2 μl-cDNA synthesis reaction volume was combined in a 100-μl final reaction volume for PCR amplification containing 0.25 μM of each oligonucleotide primer and 1.5 units of Taq DNA polymerase (Perkin-Elmer Corp.). The primer sequences were 5′-AAC- GGATCTTGATTTTTCCACGGAGCCTCTGG(FSHR-S1), 5′-TCAGAATTTCACGTGCATGGCATGCGTGATGGTA (FSH-AS2) for FSH-R and 5′-TTCGGATCTTACACATCTCTG-GAGAAGATGCACAATG(LH-R-S1), 5′-TCGAGAATT- CAGTTGAATAGCATAGGTTGATGTG(LH-R-AS2) for LH-R. Thirty cycles of PCR amplification were performed using a DNA thermal cycler (Perkin-Elmer Corp.). Each cycle consisted of a 90-s denaturation at 95°C, a 150-s annealing at 62°C, and 150 s at 70°C for enzymatic extension. After DNA amplification, the PCR mixture was extracted using phenol-chloroform, followed by ethanol precipitation. PCR-product DNA was then electrophoresed on 2% agarose gels. PCR-product gels were Southern blotted using standard methods. After alkaline denaturation and neutralization, the gels were transferred to a Nylon membrane filter. After fixation by UV irradiation, the filters were hybridized with 32P-labeled cDNA. The cDNAs were labeled with 32P by random priming and used to hybridize with DNA blots with stringent hybridization (42°C, 50% formamide) and washing (0.25× SCC, 68°C) conditions, followed by autoradiography.

Activin Assay. Tissue was homogenized in PBS. The homogenate was then centrifuged (27,000 × g for 60 min at 4°C), and the supernatant was collected for activin assay. Protein concentrations were measured using Bio-Rad reagents, a bovine IgG used as a standard. Activin A concentrations were measured by using a recently developed two-site enzyme-linked immunoabsorbent assay for activin A, previously described and validated for human serum samples (28).

Statistic Method. Comparisons between groups were performed by one-way ANOVA. The significance of differences between the mean values in the control group and each treated group were tested using Duncan’s multiple comparison test. A value of P < 0.05 was considered statistically significant.

RESULTS

RNA Expression Analysis. Northern blot analysis was performed to determine the relative abundance and size of ActR mRNA transcripts present in different ovarian epithelial cancers. As shown in Fig. 1, the results of the representative Northern blot analysis showed the presence of one ActR Ia mRNA transcript (~3 kb), two ActR IIa mRNA transcripts (~6 and 3 kb), and one ActR IIb receptor mRNA transcript (5 kb) in the total RNA prepared from the cancer tissue. The 6.0-kb Act RIa
mRNA and the 5.0-kb ActR IIb mRNA were the most abundant forms in the tissues examined. Therefore, Northern blot analysis showed substantial expression of these ActR mRNAs.

We also examined total mRNA from tissues using Northern blot analysis; normal tissues and one carcinoma showed two major transcripts of the FSH-R gene (4.1 and 2.4 kb), and normal tissues and two carcinomas showed two major transcripts of the LH-R gene (5.4 and 2.4 kb; Fig. 2). These results were further analyzed by studying the corresponding PCR-amplified FSH and LH-R cDNA obtained by reverse transcription of total RNA. Expression of FSH-R mRNA was confirmed in about half of the cancer tissues. The size of the FSH-R RT-PCR product was the same as in normal ovarian follicles (26). Similarly, LH-R mRNA expression was also detected in about two-thirds of the cancers. The two DNA fragments in the figure represent alternative splicing forms of LH-R mRNA. One, the 817-bp fragment, contains exon 9 of the human LH-R gene, and the other, the 631-bp fragment, does not (Ref. 27; Fig. 3). It has previously been shown that the human ovary expressed FSH and LH-R mRNA at relatively high levels and, consequently, RNA extracted from a sample of nonmalignant human ovary was used as a positive control. The 817-bp (FSH-R) and 817- and 631-bp (LH-R) signals are unlikely to result from amplification of contaminating genomic DNA, because the primers used were chosen to prime in what has been suggested to be separate exons. No correlation was seen between FSH-R and LH-R mRNA expression in ovarian cancer samples.

Activin was quantified by using a two-site enzyme-linked immunosorbent assay. Proteins in pre- and postmenopausal normal ovaries were extracted, and levels in the normal tissue in the ovary were around 0.59 ± 0.01 ng/mg protein (mean ± SE; n = 5). Activin production was detected in every tumor tissue examined, and the concentrations in serous adenocarcinoma (n = 9), endometrioid carcinoma (n = 4), clear cell carcinoma (n = 2), and mucinous adenocarcinoma (n = 3) were 1.8 ± 0.78, 0.96 ± 0.34, 1.2 ± 1.1, and 2.2 ± 0.13 ng/mg protein (mean ± SE), respectively (Fig. 4). Some of the serous adenocarcinoma tissue contained high activin levels, although there was high variation within these samples. Activin productions in mucinous adenocarcinoma tissue showed a tendency to be higher than those of normal tissues, but no significant difference was seen between normal and mucinous adenocarcinoma tissues.

An analysis of this data is shown in Table 1. Two pluses (+ +) were defined as those with detectable transcripts in Northern blots, whereas one plus (+) was defined as those with detectable bands only by RT-PCR, and minuses (−) were defined as those without detectable bands even by RT-PCR. Although there was no significant difference in activin concentration between the cancers expressing LH-R (+/+ +; 1.72 ± 0.61; n = 8) and those without LH-R (−; 1.88 ± 0.77; n = 9), the concentration of activin in the carcinoma expressing FSH-R (+/+ +; 2.70 ± 0.74; n = 8) was significantly higher than those of activin in the carcinoma without FSH-R (−; 0.76 ± 0.25; n = 9).

DISCUSSION

Because the actions of activin are mediated by its own receptors, which express serine/threonine kinase activity, the expression of these receptors is essential for a cell to respond to activin. Northern blot analysis in the present experiment demonstrated the presence of ActRs (type I and type II) in epithelial ovarian cancer tissue. These receptor mRNAs are similar in size to the ActR mRNAs found in other activin-responsive tissues (29–31). Taken together, the results of the present study confirm the findings of previous reports in which the expression of ActRs was examined using RT-PCR (18).
The epithelial neoplasms of the ovary are generally accepted as originating from the surface epithelium (modified mesothelium or surface celomic epithelium; Refs. 32 and 33). Several lines of evidence suggest that these ovarian epithelial tumors may be target tissues of gonadotropins: (a) binding studies (34–38) have shown a significant number of human ovarian tumors to contain binding sites for gonadotropins; (b) the growth of cell lines derived from ovarian epithelial tumors can be stimulated by gonadotropins (39, 40); and (c) ovarian tumors develop in animal models after prolonged treatment with exogenous gonadotropins or elevated levels of endogenous gonadotropins (1, 41). Although ovarian epithelial cells have not been thought to play a major role in ovarian function (i.e., steroidogenesis, follicular maturation, and so forth), a recent study demonstrated that the ovarian surface epithelium abundantly expresses FSH-R mRNA in normal tissue (13). In the present study, we found that some ovarian neoplasm originating from ovarian surface epithelium does, indeed, express an abundant level of FSH and LH-R mRNA, providing another piece of supporting evidence for the regulation of ovarian epithelial cancer cell growth by gonadotropins. This suggests that gonadotropins may be critical modulators of the development and/or progression of multiple types of ovarian tumors, although these hormones might be acting through different independent pathways depending on the cell type.

Activin synthesis has been demonstrated in normal rat and human granulosa cells, and this growth factor has been found to be capable of modulating both their proliferation and differentiation. The autocrine secretion hypothesis states that as a result of oncogene activation, neoplastic cells can escape growth-restraining mechanisms by independently producing and responding to their own growth factors. A stimulatory effect of activin on gonadal tumor cell growth was found in a previous study (42), and this finding is consistent with both the weak mitogenic action of activin on BALB/c3T3 cells (43) and its proliferation-promoting action in primary cultures of human granulosa cells (44) and erythropoietic progenitor cells (45). Mucinous adenocarcinoma was seen to secrete the highest levels of activin A in medium in a previous experiment (17), and this tumor subtype included high concentrations of activin A in our

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**Fig. 2** Northern blot analysis of gonadotropin receptor expression in human epithelial neoplasms. The RNA samples (10 μg) were prepared from isolated homogenates [N, normal (10-day follicle); S, serous adenocarcinoma; E, endometrioid carcinoma; C, clear cell carcinoma; M, mucinous adenocarcinoma] and fractionated by electrophoresis through a 1% agarose gel. The samples were blotted onto nitrocellulose membranes and covalently cross-linked using an UV cross-linker (Stratagene). Migration distances of the 28S and 18S rRNAs in parallel total RNA samples are shown. Filters were hybridized as described in “Materials and Methods.” The Northern blot shown is representative of four experiments.

**Fig. 3** Detection of the gonadotropin receptor expression using RT-PCR. RT-PCR experiments regarding human FSH and LH-R mRNA in the carcinomas [N, normal (10-day follicle); S, serous adenocarcinoma; E, endometrioid carcinoma; C, clear cell carcinoma; M, mucinous adenocarcinoma]. The PCR products were transferred to nylon membranes and subjected to Southern hybridization using human FSH and LH-R cDNA.
experiment as well. In addition, the activin concentration in serous adenocarcinoma showed high variation (from undetectable to 7.0 ng/mg protein). These data are consistent with the fact that clinically, serous adenocarcinoma is one of the most variant types of ovarian carcinomas.

Inhibins and activins are members of the transforming growth factor β superfamily of polypeptides that have been shown to have both growth-promoting and growth-inhibiting properties (46–51). The importance of inhibin and its α-subunit in the regulation of stromal cell proliferation and tumor development has been recently demonstrated by using a homologous recombination to delete the α-subunit from the mouse genome (52). The loss of the inhibin α-subunit in these mice results in gonadal tumor development within 6 weeks. Interestingly, the α-inhibin-deficient mice all had extremely elevated levels of circulating activin (53). Thus, the absence of the inhibin α-subunit or overexpression and secretion of the inhibin/activin β-subunit and dimeric activin may contribute to the development of gonadal sex cord-stromal tumors. In addition, the capability of unregulated excess activin to lead to proliferative changes associated with oncogenic transformation is clearly indicated by these studies and makes this system a prime suspect in the development of several types of ovarian cancers.

In the present study, we have shown that human epithelial ovarian cancer cells also are a site of activin and its receptor synthesis using Northern blot analysis. These results may relate

### Table 1  Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age</th>
<th>Histology</th>
<th>Clinical stage</th>
<th>FSH-R&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LH-R&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Activin concentration (ng/mg protein)</th>
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<td>S₁</td>
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<td>Serous</td>
<td>2c</td>
<td>–</td>
<td>+</td>
<td>1.86</td>
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<tr>
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<td>+</td>
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<td>E₁</td>
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<td>1.91</td>
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<tr>
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<td>2.34</td>
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<sup>a</sup> The ovarian epithelial tumors were histologically typed according to the classification system of the WHO: ++, with detectable transcripts in Northern blot; +, with bands detectable only by RT-PCR; –, without bands detectable by RT-PCR.
to the production of activin A in vivo and in vitro by these tumors. The results presented herein indicate that epithelial ovarian cancer cells are able to synthesize activin and its receptor but do not elucidate the mechanisms by which activin may play a role in the abnormal proliferation of these cells. Our data suggest that quantitative differences in activin synthesis might be present in the different tumors studied. However, further investigation using a larger number of subjects is needed to correlate activin A production with epithelial tumor subtype.

Although we have not investigated the effects of activin on normal ovarian surface epithelial cells, the results of the present study support the hypothesis that potent growth factors present on the preovulatory follicle may be involved in promoting the proliferation of the epithelial cells that cover the surface of the ovary, and the subsequent development of malignant neoplasm. In vitro experiments have shown that activin A induces FSH-R in immature granulosa cells (54, 55). Because in the present experiment FSH-R expression tends to correlate with the amount of activin production in the tumor, the expression of this receptor may be due to the influence of activin contained in the tumor.

GnRH agonists are clinically used to suppress serum gonadotropin levels by down-regulating gonadotropin secretion. However, inconsistent results with respect to the suppression of ovarian epithelial cancer growth have been reported in clinical trials to assess the efficacy of GnRH agonist treatment. This may reflect the variability of levels of expression of gonadotropin receptors in ovarian epithelial cancers, as demonstrated by this study. Therefore, analysis of gonadotropin receptors in ovarian epithelial cancers might provide a useful indicator of the efficacy of GnRH agonist treatment of ovarian cancer.

In conclusion, we show that gonadotropin receptors were expressed in ovarian epithelial cancers as observed by Northern blot analysis and RT-PCR. We further suggest that activin may exert effects on FSH-R expression due to the correlation between a relatively high production of activin and expression of ActR. Patients might often show high gonadotropin levels following operation or postmenopause, and gonadotropin might affect the development and/or progression of ovarian tumors in which the gonadotropin receptor is expressed. Additional studies are, thus, required to elucidate the exact mechanism of the development of ovarian cancer. The information gleaned from this and further studies is expected to enhance our understanding of the potential role of gonadotropin in ovarian epithelial cancer.

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