Expression of the Endostatin Gene in Epithelial Ovarian Cancer

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ABSTRACT

Endostatin, a M̅ 20,000 COOH-terminal fragment of collagen XVIII, is currently in preclinical development as a novel antiangiogenic agent. The gene expression of this molecule in 23 normal ovaries with follicle or corpus luteum and in 64 cases of epithelial ovarian cancer (27 serous, 18 mucinous, 13 endometrioid, 4 clear cell, and 2 undifferentiated carcinomas) was analyzed by PCR of RNA after reverse transcription. Seven of the cases were of low malignant potential. With regard to staging, 23 cases had stage I disease, 5 had stage II disease, 29 had stage III disease, and 7 had stage IV disease. The level of endostatin gene expression was described in terms of the ratio of the relative yield of the endostatin gene to that of the β2-microglobulin gene. Endostatin gene expression in ovarian cancers (median, 0.14; range, 0.02–1.11) was significantly higher than that in normal ovaries with follicle or corpus luteum (median, 0.08; range, 0.03–0.26; P = 0.009). International Federation of Gynecology and Obstetrics stage (P = 0.009) and residual tumor (P = 0.005) were significantly associated with endostatin gene expression; however, other clinicopathological features (e.g., patient age at diagnosis, histological subtype, and histological grade) were not significantly associated with endostatin gene expression. Survival data were available for all patients. Univariate Cox regression analysis showed the prognosis of the patients with high endostatin gene expression [equal to or greater than the median (≥0.14)] to be significantly worse than that of patients with low endostatin gene expression [less than the median (<0.14); P = 0.044]. Our results with regard to the gene expression of this endogenous inhibitor of angiogenesis present a new insight to understand the biology of epithelial ovarian cancer and may lead to the development of a new therapeutic strategy for epithelial ovarian cancer.

INTRODUCTION

For many years, the combination of cyclophosphamide–cisplatin was used as the standard of care for the treatment of ovarian cancer; then, however, a large randomized study proved that a combination of paclitaxel and cisplatin resulted in a better response rate and an improvement in progression-free interval and survival (1). Recently, Neijt et al. (2) reported that paclitaxel-carboplatin is a feasible regimen for outpatients with ovarian cancer and has a better toxicity profile than paclitaxel–cisplatin. However, they could not draw any conclusions about the efficacy of the two regimens in comparison with each other. The overall 5-year survival rate for patients with FIGO stage IIB to IV disease treated by this new chemotherapeutic regimen was about 30% (2). The early stages of malignant growth in the ovary do not usually produce symptoms, and late diagnosis is probably the main reason for the poor prognosis. An improved understanding of the mechanisms regulating the growth of ovarian cancer cells may eventually lead to techniques that facilitate early diagnosis, establishment of prognosis, or determination of response to therapy.

The growth and progression of tumors is dependent on the process of angiogenesis. This process begins when a pinpoint colony of tumor cells expands to a size where simple diffusion of nutrients (and wastes) is insufficient. New capillaries are elicited, and the tumor then enters a phase in which perfusion becomes the mechanism by which nutrients arrive and metabolic wastes are carried away (3). Therefore, blood supply is a critical factor for the growth and progression of the tumor. Recently, we have reported that gene expression of TP [an angiogenic factor (4, 5)] and AM [a vasodilatory peptide (6)] assessed by RT-PCR was significantly associated with poor prognosis in patients with epithelial ovarian cancer. The development of antiangiogenic therapy is anticipated in epithelial ovarian cancer.

Endostatin, a M̅ 20,000 COOH-terminal fragment of collagen XVIII, is currently in preclinical development as a novel antiangiogenic agent (7). Mouse endostatin was initially isolated from the conditioned media of a murine hemangiendothelioma cell line (8). Mouse endostatin, a potent inhibitor of angiogenesis in vitro and in vivo, has demonstrated potent antitumor activity in vivo without the development of resistance (8, 9). Human serum and tissue forms of endostatin have also been identified (10, 11), and the inhibitory effect of endostatin on angiogenesis has been reported previously (12). In the present study, we have examined mRNA expression of endostatin using RT-PCR in 23 ovaries with follicle or CL and 64 cases of epithelial ovarian cancer. The gene expression of this endogenous inhibitor of angiogenesis has been related to clinical and pathological parameters to further evaluate the role of endostatin in epithelial ovarian cancer.

Received 3/5/01; revised 5/15/01; accepted 5/16/01.

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2 The abbreviations used are: FIGO, International Federation of Gynecology and Obstetrics; CL, corpus luteum; RT-PCR, reverse transcription-PCR; β2-MG, β2-microglobulin; TP, thymidine phosphorylase; AM, adrenomedullin; LMP, low malignant potential.
MATERIALS AND METHODS

Patients. The 23 patients that had normal ovaries with follicle or CL ranged in age from 21–50 years (median age, 43 years), and all subjects had regular menstrual cycles. The final diagnoses were myoma uteri and/or adenomyosis (n = 14), cervical cancer stage Ib (n = 2), serous cystadenoma (n = 4), mucinous cystadenoma (n = 2), and hemorrhagic CL (n = 1).

Patients were selected from those with epithelial ovarian cancer treated between October 1989 and December 1999 at the Shimane Medical University Hospital (Izumo, Japan). Eligible patients had a histological diagnosis of primary epithelial ovarian cancer and were suitable for adequate surgical staging. Patients were excluded from this study if fresh, surgically resected specimens could not be obtained or were excluded preoperatively if they had undergone any therapy, had multiple cancers, or had severe complications. All research was conducted with patients’ informed consent and with the approval of the hospital research ethics board. The median age of the 64 eligible patients was 54 years (range, 19–87 years). Twenty-six of them were premenopausal. The patients were staged according to the 1987 criteria recommended by FIGO (13). There were 23 stage I patients, 5 stage II patients, 29 stage III patients, and 7 stage IV patients. The staging system defined by FIGO, as described elsewhere (4, 6), assumes that an adequate staging operation has been performed. Tumors were classified histologically according to the WHO criteria (14) as serous (n = 27), mucinous (n = 18), endometrioid (n = 13), clear cell (n = 4), and undifferentiated (n = 2). The tumors were classified histologically as either having LMP (n = 7) or being well differentiated (n = 13), moderately differentiated (n = 24), or poorly differentiated (n = 20 (15)).

The surveillance for recurrent disease usually consisted of physical examination, Papanicolaou smear, and serology with tumor marker examination (e.g., CA 125, CA 19-9, carcinoembryonic antigen, sialyl Tn) every month for the first year, every 2 months for the second and third year, and every 3 months for the fourth and fifth year. After 5 years, the patients were examined semiannually. A chest radiograph and computed tomography scan or ultrasonography were obtained every 6 months for 5 years after surgery and every year thereafter; if necessary, magnetic resonance imaging was performed. Recurrent disease was proven pathologically, radiographically, or serologically. Follow-up information was obtained from medical records, letter or telephone contact with patients, and information from the referring physician. Survival data were available for all patients (median, 24 months; range, 2–120 months).

MATERIAL AND METHODS

RT-PCR. RT-PCR for determination of endostatin gene expression was performed according to the method described previously (4–6). Total RNA was isolated from frozen tissue using a commercially available extraction method (Isogen; Nippon Gene Inc., Tokyo, Japan). Briefly, cDNA was prepared by random priming from 500 ng of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia-LKB, Uppsala, Sweden). Primers for endostatin gene (GenBank accession number AF018081) amplification were ATGCTGACATTACCTGCTGCC (upstream) and ATGAATCGACACCCTGCTGG (downstream), and the PCR product was 172 bp. The primers were set based on the coding sequence of endostatin in human collagen XVIII (16). Moreover, primers for the non-endostatin-coding sequence (non-endostatin gene) were chosen (GenBank accession number AF018081; upstream primer, GCAGCCACAGAGATACCA; downstream primer, CACCGGCAATGTTCTCCTCT; PCR product, 167-bp fragment). PCR was carried out in a Thermal Cycler (Perkin-Elmer Cetus, Northwalk, CT) with a mixture consisting of cDNA derived from 5 ng of RNA, 10 pmol of upstream and downstream primers for the sequences of the endostatin gene, 5 pmol of primers for the β2-MG gene (GenBank accession number V00567; upstream primer, ACCCACGGAAAAGATGAG; downstream primer, ATCTTCACCTCAGATGC; PCR product, a 120-bp fragment), 200 μmol of deoxynucleotide triphosphate, and 0.1 unit of Taq DNA polymerase with reaction buffer (Life Technologies, Inc., Rockville, MD) in a final volume of 10 μl. The conditions for PCR were denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. Thirty-four cycles of PCR were performed for each specimen, and the products were separated on 9% polyacrylamide gels. The bands were then visualized by ethidium bromide staining. NIH analysis software version 1.61 (NIH, Bethesda, MD) was used to scan the RT-PCR polyacrylamide gels after photographic documentation. The software measures relative mean density over a fixed gray scale range after correction for background. The endostatin expression was described in terms of the relative yield of the endostatin gene to that of the β2-MG gene.

Statistical Analysis. Mann-Whitney U test and Kruskal-Wallis one-way ANOVA by ranks were used as appropriate for evaluation of differences between end points. The Cox proportional hazards model was used in survival analysis. Maximum likelihood parameter estimates and likelihood ratio statistics in the Cox proportional hazards models were obtained with the use of a statistical package, EPICURE (17). Kaplan-Meier curves were compared by the univariate Cox regression analysis. All Ps presented were two-sided. \( P < 0.05 \) was considered significant.

RESULTS

RT-PCR and Endostatin and Non-endostatin Gene Expression. To determine the number of PCR cycles appropriate for quantification, PCR was performed from 22–40 cycles at an increment of 2 cycles. The expression ratios of endostatin and non-endostatin to \( \beta_2 \)-MG were reasonably constant from 24–34 cycles, respectively (data not shown). Therefore, in the subsequent experiments, the values at 30 PCR cycles for subsequent analysis. This method has been described in the previous investigation (4–6).
Endostatin gene expression was defined as the expression of target genes. The representative profile of endostatin gene expression by RT-PCR is shown in Fig. 1. Furthermore, the sequences of PCR products were analyzed, and they were identical to the sequence of the endostatin and non-endostatin gene, respectively. There was no significant correlation between the expression of the endostatin and non-endostatin gene in normal ovaries (P = 0.208) and ovarian cancer (P = 0.082), respectively. Therefore, the endostatin gene expression obtained from RT-PCR in this investigation seemed to be independent of collagen XVIII gene activity.

**Endostatin Gene Expression and Clinicopathological Features.** Endostatin gene expression of ovarian cancers (median, 0.14; range, 0.02–1.11) was significantly higher than that of normal ovaries with follicle or CL (median, 0.08; range, 0.03–0.26; P = 0.009). No significant difference was noted between ovaries with follicle versus CL in terms of endostatin gene expression (P = 0.564). The gene expression values are mean values from at least three independent RT-PCR experiments. The values of endostatin gene expression in ovarian cancers are classified according to patient age at diagnosis, stage of disease, residual tumor mass after initial surgery, histological subtype, and grade (Table 1). FIGO stage (P = 0.009) and residual tumor (P = 0.005) were significantly associated with endostatin gene expression.

**Endostatin Gene Expression and Prognosis.** In a follow-up study of 42 cases after complete resection of the primary tumors by surgical operation, endostatin gene expression of the 9 patients who experienced recurrence (median, 0.10; range, 0.02–0.59) was statistically the same as that of 33 patients without recurrence (median, 0.24; range, 0.04–1.44). As shown in Fig. 2, we found the prognosis of the patients with high endostatin gene expression [equal to or greater than the median (≥0.14)] to be significantly worse than that of patients with low endostatin gene expression [less than the median (<0.14)] by univariate Cox regression analysis (P = 0.044). Moreover, FIGO stage (III-IV; P = 0.0006) and residual disease (≥2 cm; P = 0.001) were found to be significantly associated with a poor prognosis in univariate Cox regression analysis (Table 2). Serious tumors and poorly differentiated tumors are generally thought to be more aggressive than other histological subtypes and other grades, respectively (18). However, such findings were not noted in this study (Table 2). Multivariate Cox regression analysis revealed that FIGO stage III–IV is an independent prognostic factor (P = 0.038; Table 3).

**DISCUSSION**

Collagen XVIII is an extracellular matrix protein identified by cDNA cloning (19, 20). The function and significance of this molecule is not known, but a M₇ 20,000 proteolytic fragment called endostatin that is released from the COOH terminus of the collagen XVIII chain has been shown to inhibit endothelial cell proliferation, angiogenesis, and tumor growth (8). The regulation of angiogenesis is thought to be controlled by a balance of stimulators and inhibitors (21). Many of the angiogenesis inhibitors identified to date are fragments of large molecules. Recently, Wen et al. (22) suggested that the generation of endostatin from collagen XVIII is at least a two-step process, involving a metal-dependent early step and an elastase activity-dependent final step based on the cell culture study of hemangiendothelioma. In this study, endostatin gene expression of ovarian cancers was significantly higher than that of normal ovaries with follicle or CL. This might reflect a biological difference between physiological and pathological angiogenesis in the generation activity of endostatin from collagen XVIII.

The range of endostatin gene expression of ovarian cancers was larger than that of normal ovaries. The generation activity of endostatin from collagen XVIII might also differ according to the state of pathological angiogenesis each epithelial ovarian cancer bears.

Many locally advanced human cancers are resected for apparent cure after the usual systemic work-up for metastases proves negative. However, explosive metastatic recurrence after such resection for advanced disease is not an uncommon clinical observation (23). This clinical observation has been recreated in animal tumor models, in which the growth of small lesions is suppressed in the presence of a large primary tumor and is termed concomitant tumor resistance (24, 25). O’Reilly et al. (26) have proposed that a primary tumor, which is capable of stimulating angiogenesis in its own vascular bed, simultaneously inhibits angiogenesis in vascular beds of secondary, metastatic lesions. Circulating factors produced by the primary tumor are responsible for the suppression of distant tumor growth. One of these factors is endostatin, which was identified from the urine of mice bearing Lewis lung carcinoma (8).

**Table 1** Clinicopathological features and endostatin gene expression

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Median (range)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age at the time of diagnosis</td>
<td></td>
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<tr>
<td>≤50 yrs (n = 26)</td>
<td>0.13 (0.02–0.83)</td>
<td>0.984</td>
</tr>
<tr>
<td>&gt;50 yrs (n = 38)</td>
<td>0.14 (0.03–1.11)</td>
<td></td>
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<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
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<tr>
<td>I–II (n = 28)</td>
<td>0.10 (0.02–0.60)</td>
<td>0.009</td>
</tr>
<tr>
<td>III-IV (n = 36)</td>
<td>0.17 (0.04–1.11)</td>
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<tr>
<td>Residual disease</td>
<td></td>
<td></td>
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<tr>
<td>≤2 cm (n = 42)</td>
<td>0.10 (0.02–1.04)</td>
<td></td>
</tr>
<tr>
<td>&gt;2 cm (n = 22)</td>
<td>0.22 (0.04–1.11)</td>
<td></td>
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<tr>
<td>Histological subtype</td>
<td></td>
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</tr>
<tr>
<td>Serous (n = 27)</td>
<td>0.16 (0.03–1.04)</td>
<td>0.879</td>
</tr>
<tr>
<td>Mucinous (n = 18)</td>
<td>0.10 (0.04–1.11)</td>
<td></td>
</tr>
<tr>
<td>Endometrioid (n = 13)</td>
<td>0.14 (0.04–0.83)</td>
<td></td>
</tr>
<tr>
<td>Clear cell (n = 4)</td>
<td>0.06 (0.03–0.60)</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP (n = 7)</td>
<td>0.10 (0.03–0.22)</td>
<td>0.335</td>
</tr>
<tr>
<td>Well differentiated (n = 13)</td>
<td>0.16 (0.05–0.60)</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated (n = 24)</td>
<td>0.14 (0.04–0.83)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated (n = 20)</td>
<td>0.16 (0.02–1.11)</td>
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We noted that FIGO stage and residual tumor were significantly associated with endostatin gene expression. This finding indicates that there might be a possible release of an inhibitory effect that the primary tumor has over disseminated tumor cells in epithelial ovarian cancer. Moreover, elevated endostatin gene expression is significantly correlated with reduced survival when examined by univariate analysis. The principle of our treatment was primary cytoreductive surgery before chemotherapy. The ovarian cancer might tend to undergo compensatory growth after tumor resection due to the loss of inhibitor factor, i.e., endostatin. Consequently, therapy that reduces the tumor mass may tend to accelerate the growth of the remaining tumor and tumor metastases. However, this speculation is putative because multivariate analysis demonstrated that endostatin gene expression is not an independent prognostic factor among the clinicopathological parameters studied. Moreover, it has to be noted that the number of cases was limited.

TP and AM gene expression was measured in 34 and 47 of 64 patients, respectively. TP and AM gene expression, as assessed by RT-PCR, was significantly associated with poor prognosis in patients with epithelial ovarian cancer, respectively (4–6). There was no significant correlation between TP and endostatin gene expression (P = 0.789) and AM and endostatin gene expression (P = 0.411). Endostatin might affect the worse prognosis independently of TP and AM. However, this speculation leaves much to be investigated.

The RT-PCR method we used for determination of endostatin gene expression is convenient because it does not require radioisotopes or relatively large amounts of tumor tissue. Because even a small amount of samples obtained from specimens during operation is sufficient for the evaluation of endostatin gene expression, RT-PCR detection of endostatin gene expression may make it possible to identify epithelial ovarian cancer patients with poor prognoses before chemotherapy. In those patients, it is anticipated that administration of an angiogenic inhibitor like endostatin would be applied in conjunction with the conventional cytotoxic chemotherapy. Indeed, i.m. administration of the formulated endostatin gene inhibited both the growth of primary tumors and the development of metastatic lesions in murine models (27). Moreover, it has been revealed that rat endostatin is a potent anticancer agent in a carcinogen-induced, spontaneously arising rat breast cancer model (28).
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