Clinical implications of expression of cyclooxygenase-2 related to angiogenesis in ovarian cancer

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Abstract. Angiogenesis is essential for the development, growth and advancement of solid tumors. Cyclooxygenase (cox)-2 is recognized as an angiogenic factor in various tumors. This prompted us to study the clinical implications of cox-2 expression and angiogenesis in ovarian cancer. There was a significant correlation between microvessel counts and cox-2 levels. Cox-2 localized in the cancer cells, but not in the stromal cells of ovarian cancer tissue. Cox-2 levels increased with the advancement, and the prognosis of the 30 patients with high cox-2 expression was extremely poor (33%), while the 24-month survival rate of the other 30 patients, those with low cox-2 expression, was 67%. Furthermore, cox-2 levels significantly correlated with VEGF levels. VEGF associated with cox-2 might work on angiogenesis with advancement. Therefore, long-term administration of cox-2 inhibitors might be effective on the suppression of regrowth or recurrence after intensive treatment for advanced ovarian cancer.

Introduction

Angiogenesis is essential for the development, growth and advancement of solid tumors (1). Angiogenic factors from tumors induce and activate matrix metalloproteinase, plasminogen activator, collagenase and other enzymes in endothelial cells. The enzymes dissolve the basement membrane of endothelial cells, after which the endothelial cells proliferate and migrate under the influence of angiogenic factors. Angiogenic factors induce the production of integrins in endothelial cells. The endothelial cells then form immature capillary tubes. Specific angiogenic factors show specific angiogenesis in each tumor. The angiogenic factors, vascular endothelial growth factor (VEGF) (2-6), thymidine phosphorylase (TP) identified with platelet-derived endothelial cell growth factor (7-11), interleukin (IL)-8 (12,13) and basic fibroblast growth factor (bFGF) (14-16), along with the angiogenic transcription

factor (ETS-1) (17-20), work on angiogenesis in gynecological cancers, especially in ovarian cancer (5,6,11,16) as follows.

TP remarkably highly expressed in some endometrioid carcinomas and serous cystadenocarcinomas of the ovary and in some stage III and IV ovarian cancers, and works on advancement as an angiogenic factor (11). Basic FGF highly expressed in advanced ovarian cancer, regardless of histological type (16). $VEGF_{165}$ was elevated in all stages of ovarian cancer via angiogenic activity, regardless of histopathological type (5). Furthermore, a significantly increased VEGF level from the primary tumor to the peritoneal disseminated lesion of ovarian cancer was found in 8 of 30 cases. The 24-month survival rate of patients with a significantly increased VEGF level was extremely poor (0%) in comparison with that of patients with no change in the level (68%) from the primary tumor to the peritoneal disseminated lesion. Therefore, VEGF may be the main contributor to the advancement of ovarian cancer, especially in peritoneal disseminated lesions, and VEGF level is available as a prognostic indicator (6).

Recently, it has been reported that cox-2 works on angiogenesis associated with tumor growth and advancement in the following tumors: advanced ovarian serous carcinomas (21), uterine endometrial cancer (22), breast cancer (23), gastric cancer (24), renal cell carcinomas (25), head and neck squamous cell carcinoma (26) and colon cancer (27). Colon cancer cell line HCA-7 cells overexpress cox-2 and co-express VEGF, bFGF, transforming growth factor (TGF)-ß and platelet-derived growth factor (PDGF) (27). The metabolites of cox-2 prostaglandin (PG)E1 and PGE2 have weak angiogenic activity (28), and PGE1 and PGE2 induce VEGF production in osteoblasts, synovial fibroblasts and macrophages (29-31). This prompted us to study the protein expression of cox-2 and various angiogenic factors along with the various clinical backgrounds of ovarian carcinoma patients in order to ascertain the clinical implications of cox-2 in various ovarian cancers.

Materials and methods

Patients. Prior informed consent for the following studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty patients ranging from 34 to 82 years of age with ovarian cancer (stage I, 20 cases; stage II, 20 cases; stage III, 20 cases; including 14 cases of serous cystadenocarcinoma, 10 cases of serous papillary cystadenocarcinoma, 16 cases of mucinous cystadenocarcinoma, 12 cases of endometrioid adeno-

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carcinomas, and 8 cases of clear cell adenocarcinoma) underwent curative surgery at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between December 1999 and January 2003. None of the patients had received any therapy before ovarian cancer tissue was taken. A part of each ovarian cancer tissue was snap-frozen in liquid nitrogen to determine cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF, VEGF and TP levels, and a neighboring part was submitted for histopathological study including immunohistochemical staining for cox-2 and factor-VIII-related antigen. The clinical stage of ovarian cancer was determined using the International Federation of Obstetrics and Gynecology (FIGO) classification (32).

Immunohistochemistry. Sections (4-μm) of formalin-fixed paraffin-embedded tissue of uterine endometrial cancer were cut with a microtome and dried overnight at 37˚C on a silanized-slide (Dako, Carpinteria, CA, USA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. Immunohistochemical staining for factor-VIII-related antigen, which is synthesized by vascular endothelial cells, is specific for the endothelial cells of blood vessels (33) and is useful for detecting tumor angiogenesis (34). The samples for cox-2 antigen were soaked in a citrate buffer, and then microwaved at 100˚C for 10 min, and those for factor-VIII-related antigen and CD34 were treated with 0.3 μg/ml trypsin in phosphate buffer at room temperature for 20 min. The protocol for a Dako LSAB2 Kit peroxidase (Dako) was followed for each sample. In the described procedures, rabbit anti-human cox-2 (Cayman Chemical, Ann Arbor, MI, USA), rabbit anti-factor VIII-related antigen (Zymed, San Francisco, CA, USA) and mouse CD34 (Dako) were used at dilutions of 1:25, 1:2 and 1:40, respectively, as the first antibodies. The addition of the first antibody was omitted from the protocols for negative controls of cox-2, factor-VIII-related antigen or CD34, respectively.

Vessels were counted in the five highest density areas at x200 magnification (using a combination of 20 x objective and 10 x ocular, 0.785 mm² per field) by blinded investigators. Microvessel counts were expressed as the mean numbers of vessels in the five highest density areas (35). Microvessel density was evaluated by the counting of microvessels.

Enzyme immunoassay for determination of Cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF, VEGF and TP antigens. All steps were carried out at 4˚C. Ovarian cancer tissue (wet weight: 10-20 mg) was homogenized in HG buffer (5 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM ethyleneglycol-bis-[ß-aminoethyl ether]-N,N,N',N'-tetraacetic acid, 1 mM $MgCl_2$, 2 mM dithiothreitol, 25 μg/ml aprotinin and 25 μg/ml leupeptin) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12,000 rpm for 3 min to obtain the supernatant. The protein concentration of samples was measured by the method of Bradford (36) to standardize cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF, VEGF and TP antigen levels.

Cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF and VEGF antigen levels in the samples were determined by a sandwich enzyme immunoassay using a Human COX-2 EIA kit (Immuno-Bio-
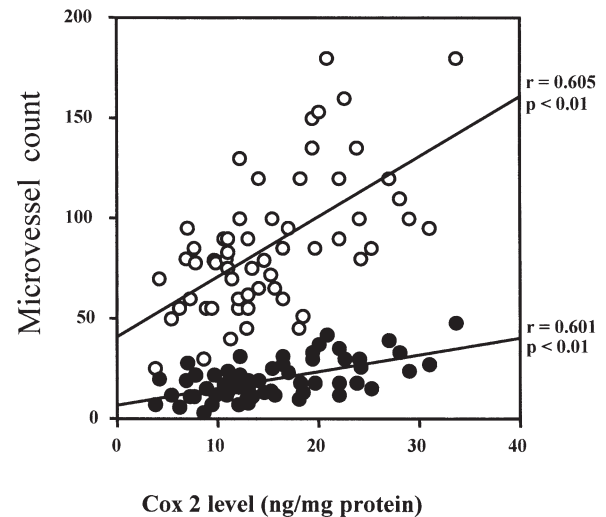

Figure 1. Correlation between microvessel count and cox-2 in ovarian cancer. Closed circles, microvessel counts by immunohistochemical staining for factor-VIII-related antigen (MVC-F8); open circles, microvessel counts by immunohistochemical staining for CD34 (MVC-CD34).

logical Laboratories, Gunma, Japan), Human IL-1α Quantikine (R&D Systems, Minneapolis, MN, USA), Human IL-1ß Quantikine (R&D Systems), Human TNF-α Quantikine (R&D Systems), Human IL-8 Quantikine (R&D Systems), Human bFGF Quantikine (R&D Systems) and a Human VEGF assay kit-IBL (Immuno-Biological Laboratories), respectively. TP antigen levels were determined by the method described by Nishida *et al* (37). The levels of cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF, VEGF and TP were standardized with the corresponding cellular protein concentrations.

Statistics. Cox-2, IL-1α, IL-1ß, TNF-α, IL-8, bFGF, VEGF and TP levels were measured from three parts of the same tissue in triplicate. Statistical analysis was performed using the Student's t-test. Differences were considered significant when p<0.05. Correlation evaluations between microvessel counts and cox-2 levels and between VEGF and cox-2 levels were analyzed by Pearson's product-moment correlational coefficient. Positive correlation was considered significant when p<0.05.

Results

There was a significant correlation between microvessel counts by immunohistochemical staining for factor-VIII-related antigen (MVC-F8) and CD34 (MVC-CD34) and cox-2 levels (r=0.601, p<0.01 and r=0.605, p<0.01, respectively), as shown in Fig. 1, and also between MVC-F8/MVC-CD34 and VEGF (r=0.472, p<0.01/r=0.477, p<0.01), between MVC-F8/MVC-CD34 and bFGF (r=0.619, p<0.01/r=0.583, p<0.01) and between MVC-F8/MVC-CD34 and TP levels (r=0.655, p<0.01/r=0.548, p<0.01), but not between MVC and IL-1α, IL-1ß, TNF-α or IL-8 levels (data not shown) in ovarian cancer. Cox-2 diffusely localized in the cancer cells, but not in the stromal cells of ovarian cancer tissue in all cases given, as shown in Fig. 2 for a representative case; a 46-year-old with a stage Ia, serous cystadenocarcinoma.
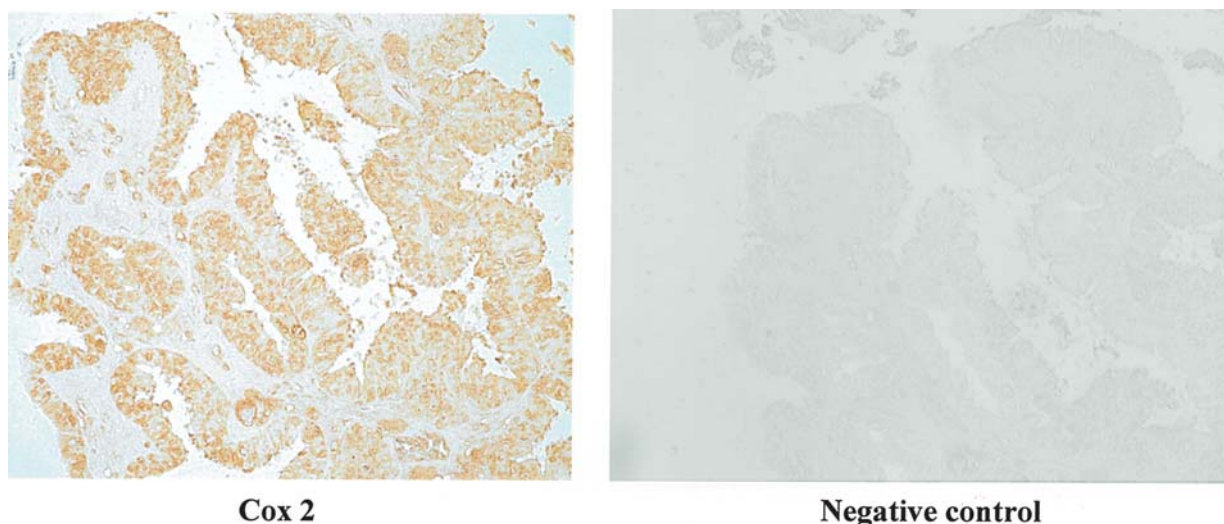
Cox 2

Negative control

Figure 2. Immunohistochemical staining for cox-2 in ovarian cancer (x100, original magnification). A representative case of serous cystadenocarcinoma of the ovary. Rabbit anti-human cox-2 (Cayman Chemicals) was used at a dilution of 1:25 as the first antibody. Dark brown staining represents positive for cox-2 antigen.
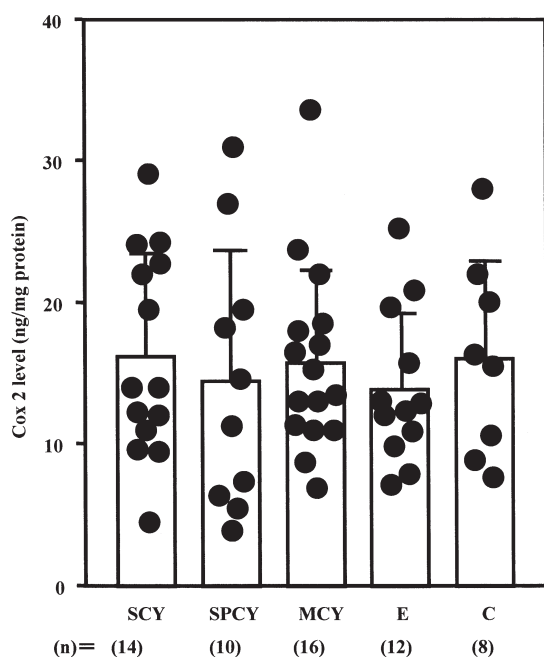

Figure 3. Levels of cox-2 in ovarian cancer, classified according to histopathological type. SCY, serous cystadenocarcinoma; SPCY, serous papillary cystadenocarcinoma; MCY, mucinous cystadenocarcinoma; E, endometrioid adenocarcinoma; C, clear cell adenocarcinoma. Each level is the mean of 9 determinations.
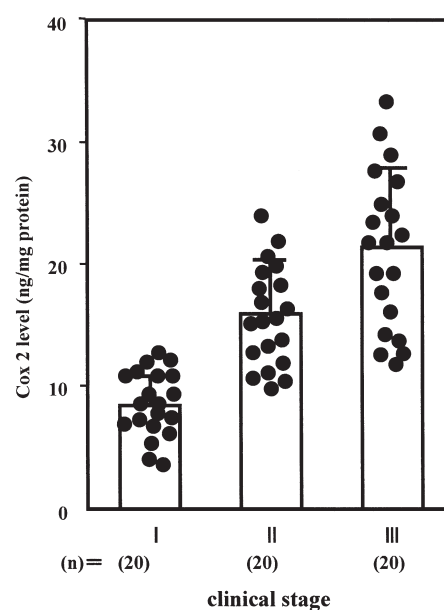

Figure 4. Levels of cox-2 in ovarian cancer, classified according to clinical stage. Clinical stages of ovarian cancer are according to FIGO. Each level is the mean of 9 determinations. [*]p<0.0001.

Among histopathological types, cox-2 expression did not demonstrate any specific relationship, as shown in Fig. 3. Cox-2 levels significantly increased with advancement (between stages I and II, p<0.0001; between stages II and III, p<0.0001; and between stages I and III, p<0.0001) as shown in Fig. 4. The prognosis of the 30 patients with high cox-2 expression was extremely poor (33%), while the 24-month survival rate of the other 30 patients, those with low cox-2 expression, was significantly better (67%), as shown in Fig. 5. Furthermore, cox-2 levels significantly (r=0.509, p<0.01)

correlated with VEGF levels, as shown in Fig. 6., but not with IL-1α, IL-1ß, TNF-α, IL-8, bFGF and TP levels (data not shown).

Discussion

Among angiogenic factors, VEGF has been evaluated as an important factor for tumor angiogenesis, which is essential for the growth of solid tumors. Generally speaking, VEGF secreted from tumors contributes to tumor growth, not via an autocrine pathway to tumor cells but via a paracrine pathway to surrounding microvessels (38). The elevation of VEGF in ovarian cancer, especially in peritoneal disseminated lesions,
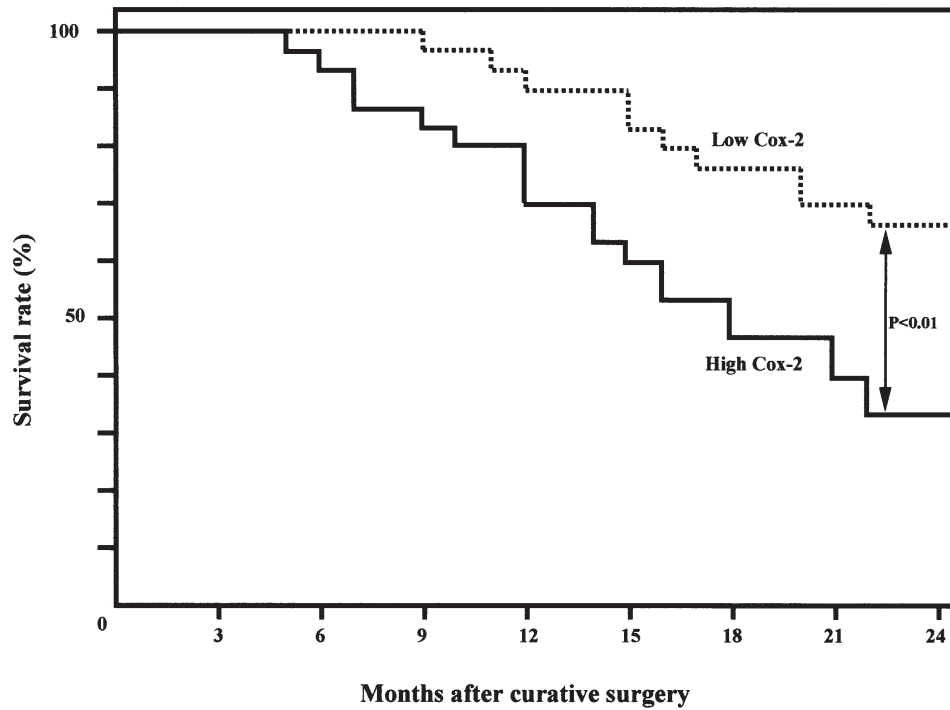
Figure 5. Survival rate after curative resection for ovarian cancers. Patient prognosis was analyzed with a 24-month survival rate. High cox-2, cases with high cox-2 levels (>14 ng/mg protein), n=30; Low cox-2, cases with low cox-2 levels (<14 ng/mg protein), n=30.
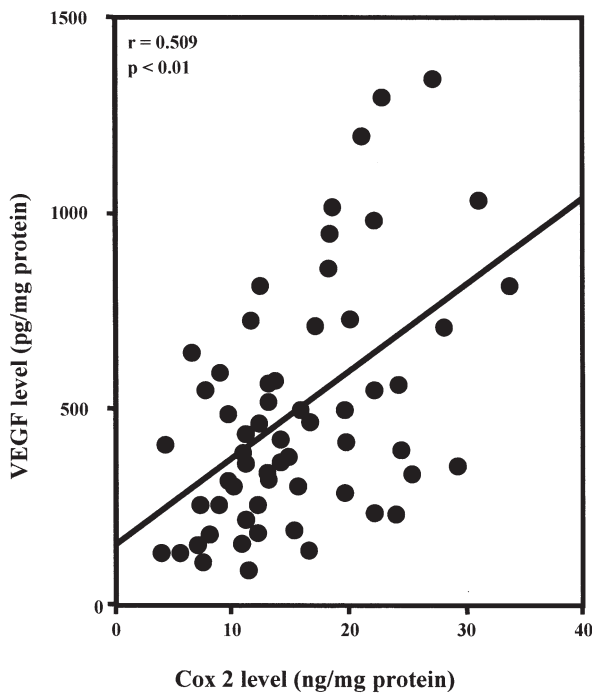

Figure 6. Correlation between cox-2 and VEGF in ovarian cancers.

correlates with a worse patient prognosis (5,6). On the other hand, KDR, a receptor of VEGF, is expressed by some ovarian cancer cells that coexpress VEGF (39). Coexpression of VEGF and KDR by tumor cells in ovarian cancer raises the possibility of autocrine stimulation (39).

It has been reported that cox-2 might work on an angiogenic mediator without any interaction of angiogenic factors,

including VEGF, only in serous carcinoma of the ovary (21). In the present study, positive correlation of cox-2 with micro-vessel density indicates that cox-2 might be a candidate angiogenic mediator in ovarian cancer. Positive correlation of VEGF with cox-2 shows that the angiogenic potential of VEGF might be supported by cox-2 in cancer cells. Cox-2 expression was up-regulated with advancement, which indicates that VEGF interacting with cox-2 might work on angiogenesis throughout the advancement of ovarian cancer regardless of histopathological type. Furthermore, it was demonstrated that the prognosis of patients with high cox-2 linked to high VEGF was extremely poor in comparison with that of patients with low cox-2 linked to low VEGF.

The basic problem in treating ovarian cancer is that it is not easy to discover it at an early stage and accomplish complete (microscopic) curative resection. A critical strategy to obtain a better patient prognosis for advanced ovarian cancer patients must achieve macroscopically complete removal of disseminated tumors, integrated with corresponding intensive chemotherapy, and then must accomplish long-term suppression of ovarian cancer regrowth or recurrence. After such a treatment of intensive surgery and chemotherapy, it is difficult to determine an appropriate schedule for additional chemotherapy. Therefore, a long-term alternative treatment, as a prophylactic approach to suppress regrowth or recurrence without any severe side effects, should be considered. Based on the present study, inhibition of cox-2 might lead to the suppression of VEGF expression in ovarian cancer. The cox inhibitor, ibuprofen, inhibited angiogenesis in rat glioma (40). A selective cox-2 inhibitor (NS-398) and aspirin inhibited the production of VEGF, bFGF, TGF-ß and PDGF and the stimulation of endothelial migration and tube formation in cox-2 overexpressing cells (27). Therefore, long-term

administration of cox-2 inhibitors might be effective on the suppression of regrowth or recurrence after intensive treatment for advanced ovarian cancer, regardless of histopathological type.

Acknowledgements

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