Role of protease activated receptor-2 in tumor advancement of ovarian cancers


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Background: Protease activated receptor-2 (PAR-2) has been implicated in cellular proliferation, invasion and metastasis with angiogenesis in various tumors. This prompted us to study the role of PAR-2 in tumor advancement of ovarian cancers.

Materials and methods: Forty-eight patients underwent surgery for ovarian cancers. In ovarian cancers, PAR-2 histoscores and mRNA levels were determined by immunohistochemistry and real-time reverse transcription-polymerase chain reaction, respectively. Patient prognosis was analysed with a 36-month survival rate. Microvessel counts were determined by immunohistochemistry for CD31 and factor VIII-related antigen and the rate of cell proliferation was determined by immunohistochemistry for Ki67.

Results: Immunohistochemical staining revealed distribution of PAR-2, dominantly in cancer cells and faintly in stromal cells of the tumor. PAR-2 histoscores in cancer cells and mRNA levels both significantly increased in ovarian cancers with clinical stages (I < II < III < IV, P < 0.05), regardless of histopathological type. The 36-month survival rate of 24 patients with high PAR-2 was poor (58%), while that of the other 24 patients with low PAR-2 was significantly higher (83%). There were significant correlations between PAR-2 histoscores in cancer cells and mRNA levels with microvessel counts and with the rate of cell proliferation in ovarian cancers.

Conclusions: PAR-2 was up-regulated during ovarian cancer progression. Therefore, PAR-2 might work on tumor advancement of ovarian cancers via angiogenic activity and is considered to be a novel prognostic indicator in ovarian cancers.

Key words: PAR-2, ovarian cancer, angiogenesis, prognostic indicator, tumor advancement

introduction

While the involvement of soluble and matrix-immobilized protease during basement membrane degradation is well recognized, the role of enzymatically activated cell surface receptors has not been elucidated. Protease activated receptors (PARs), a family of four 7-transmembrane G-protein coupled receptors, are activated by serine proteases [1]. Macromolecular assembly and generation of serine proteases on cellular surfaces are largely involved in the regulation of the metastatic cascade [2]. It is postulated that the PAR family system may simply provide redundancy in a pathway important to the regulation of various biological processes. Most importantly, it may provide a framework to define the role of distinct PARs in angiogenesis and tumor metastasis. The second member of the PAR family, PAR-2, is activated mainly by trypsin-like proteases [1]. The gene encoding human PAR-2 was isolated from a human genomic cDNA library using hybridization to a probe derived from the 3’ exon of the mouse PAR-2 gene [3] and subsequently cloned from human kidney cDNA [3, 4] and that was localized to chromosome 5q13 [5].

PAR-2 is expressed in the gastrointestinal tract, pancreas, kidney, liver, lung, vasculature, eye, prostate, ovary and uterus [4, 6]. PAR-2 expression has also been observed in cancers of the lungs [7], liver [8], prostate [4, 9], thyroid [8], breast [8, 10], stomach [11–13], colon [14, 15], pancreas [16, 17], gallbladder [18], and melanoma [9] and glioblastoma [19]. In gastric cancer cell line MKN-1, trypsin stimulates an integrin α5β1-dependent adhesion to fibronectin and proliferation through PAR-2 [13]. In lung cancers, PAR-2 mRNA expression increased 16-fold in pulmonary tumor alveolar walls, compared with in normal alveolar tissues [7]. There is an up-regulation of PAR-2 in proliferating stromal fibroblasts surrounding the carcinoma cells in breast cancers [8]. PAR-2 promotes tumor cell proliferation of the colon [15, 14], gastrin [12, 13], pancreas [16, 17] and glioblastoma [19]. To elucidate the role of PAR-2 in ovarian cancers, we analysed the manner of PAR-2 expression in ovarian cancers according to clinical backgrounds.

materials and methods

patients and tissues

Prior informed consent for the following studies was obtained from all patients and approval was given by the Research Committee for Human Subjects, Gifu University School of Medicine. Forty-eight patients (11 stage
I cases, seven stage II cases, 22 stage III cases and eight stage IV cases; and 12 cases of serous papillary cystadenocarcinoma, 10 cases of serous cystadenocarcinoma, 13 cases of mucinous cystadenocarcinoma, seven cases of clear cell adenocarcinoma and six cases of endometroid adenocarcinoma) ranging from 26 to 83 years of age underwent resection for ovarian cancers at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between March 1998 and September 2002. Patient prognosis was analysed in relation to 36-month survival rate. None of the patients had received any pre-operative therapy. The tissues of ovarian cancer were obtained immediately after surgery. The tissues for RNA isolation were snap-frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinical staging of ovarian cancers was determined by International Federation of Gynecology and Obstetrics (FIGO) classification [20].

**immunohistochemistry**

Four-μm sections of formalin-fixed paraffin-embedded tissue samples from ovarian cancers were cut with a microtome and dried overnight at 37°C on a silanized-slide (Dako, Carpinteria, CA, USA). The protocol of universal Dako Labelled Streptavidin-Biotin kit (Dako, Carpinteria, CA, USA) was followed for each sample. Samples were deparaffinized in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate buffer saline (PBS). The samples were then placed in 10 mM citrate buffer (pH 6.0) and boiled in a microwave for 10 min for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H2O2 for 10 min. The primary antibodies, goat PAR-2 (C-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse CD31 (Dako, Glostrup, Denmark) and rabbit factor VIII-related antigen (Zymed Laboratories, South San Francisco, CA, USA) were used overnight at 4°C at dilutions of 1:100, 1:10 and 1:2, respectively. The slides were washed and biotinylated secondary antibody (Dako, Carpinteria, CA, USA) was applied for 30 min. After rinsing in PBS, streptavidin-conjugated horseradish peroxidase (Dako, Carpinteria, CA, USA) was added for 30 min. Slides were then washed and treated with the chromogen 3, 3′-diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min, then rinsed in PBS, and counterstained with Mayer’s haematoxylin, dehydrated in graded ethanol, cleared in xylene and cover-slipped with a mounting medium, Entellan New (Merck, Darmstadt, Germany). For confirmation of the specificity for PAR-2 antigen, we also used another PAR-2 antibody, goat PAR-2 (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the negative controls of PAR-2, CD31 and factor VIII-related antigen, the corresponding pre-immune animal sera (goat, mouse and rabbit, respectively) (Dako, Carpinteria, CA, USA) were used instead of the primary antibodies.

**assessment of histochemical score (histoscore)**

All sections of immunohistochemical staining for PAR-2 were evaluated in a semiquantitative fashion by two pathologists according to the method described by McCarty et al. [21], which considers both the intensity and the percentage of cells stained at each intensity. Intensities were classified as 0 (no staining), 1 (weak staining), 2 (distinct staining), 3 (strong staining) and 4 (very strong staining). For each stained section, a value designated histoscore was obtained by application of the following algorithm: histoscore = ∑(i × Pi), where i and Pi represent intensity and percentage of cells that stain at each intensity, respectively, and corresponding histoscores were calculated separately.

**assessment of microvessel density (mvd)**

The MVD was assessed with microvessel counts (MVCs) in sequential tissue sections stained with mouse CD31 and rabbit factor VIII-related antigen antibodies. Blood vessels with a clearly defined lumen or a well defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting [22]. Five areas of highest vascular density were chosen and microvessel counting was performed at ×200-magnification by two investigators. The MVCs were determined as the mean of the vessel counts obtained from these fields [23].

**determination of the rate of cell proliferation**

The rate of cell proliferation was evaluated using the mouse anti-human Ki67 monoclonal antibody (Dako, Carpinteria, CA, USA) directed against a nuclear antigen present in cells that are in G1, S, G2 or M phase of the cell cycle. Quiescent G0 cells are not recognized [24]. The Ki67 monoclonal primary antibody was applied to tissue at a 1:50 dilution and incubated at 4°C overnight, followed by incubation with the biotinylated secondary antibody (Dako, Carpinteria, CA, USA) for 30 min and streptavidin-conjugated horseradish peroxidase (Dako, Carpinteria, CA, USA) for 30 min. The sections were examined by light microscopy at ×400. Each section was divided into four to 10 regions; a minimum of 100 cells was counted in every region excluding degenerated and necrotic areas. The results were expressed as the mean percentage of positively stained cells in a section [25].

**preparation of standard template for real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Internal standard template for real-time PCR was produced by PCR amplification using the primers of PAR-2 gene, 511-947 in the cDNA (PAR-2-TS: 5′-GTGAGAGGTTTCATCGTG-3′ and PAR-2-TAS: 5′-CTGATCATCAGCACATAGGC-3′). The DNA template was purified using a GeneClean II kit (Qiogene, Irvine, CA, USA). The copy numbers of the standard template were determined to quantitate PAR-2 mRNA level in samples for real-time reverse transcription (RT)-PCR.

**real-time RT-PCR**

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method [26]. The total RNA (3 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 U/μl, Invitrogen, Carlsbad, CA, USA) and the following reagents: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, 0.1M dithiothreitol, 10 mM deoxynucleotide [deoxyadenosine, deoxythymidine, deoxyguanosine and deoxycytidine] tri-phosphates (dNTPs) mixture and random hexamers (Invitrogen) at 37°C for 1 hr. The reaction mixture was heated for 5 min at 94°C to inactivate MMLV-RTase.

Real-time PCR reaction was performed with a Takara Ex Taq R-PCR kit, version 1.0 (Takara, Otsu, Japan), using a smart cycler system (Cepheid, Sunnyvale, CA, USA). The reaction solution (25 μl) contained Takara Ex Taq HS (5 units/μl), 10 X R-PCR buffer, 250 mM Mg2+ solution, 10 mM dNTP mixture, SYBR Green 1 (1:1000 dilution; CambrexBio Science, Rockland Inc., Rockland, ME, USA) and 20 μM of the primers of PAR-2 gene, 622–806 in the cDNA (PAR-2-TS: 5′-AGAGGTATTGGGTCATCGTG-3′ and PAR-2-TAS: 5′-GCAGGAAATGGAAGATGTCTG-3′) with the transcribed total RNA from the tissue and a serially diluted standard template. The real-time PCR reactions were initially denatured by heating at 95°C for 30 sec, followed by 40 cycles consisting of denaturation at 94°C for 10 sec, annealing at 55°C for 5 sec and extension at 72°C for 20 sec. A strong linear relationship between the threshold cycle and the log concentration of the starting DNA copy number was always shown (correlation coefficient > 0.99). Quantitative analysis was performed to determine the copy number of each sample.

**statistical analysis**

PAR-2 mRNA levels were determined from three parts taken from each tumor, and each sample was analysed in triplicate. PAR-2 histoscores and
mRNA levels were calculated using Student’s t-test. The 36-month survival rate was calculated according to the Kaplan–Meier method, and analysed by the log-rank test. The correlations between PAR-2 histoscores and mRNA levels with MVCs, and with the rate of cell proliferation were performed by bivariate Pearson’s correlation. Differences were considered significant when \( P \) was less than 0.05.

Figure 1. Immunohistochemical staining for PAR-2 in ovarian cancers (original magnification ×200). A representative case of clear cell carcinoma of the ovary. Goat anti-human PAR-2 antibody (Santa Cruz) was used at a dilution of 1: 100 as the primary antibody. Dark brown staining represents positive for PAR-2 antigen.

Figure 2. PAR-2 histoscores in cancer cells and in stromal cells of ovarian cancers. PAR-2 histoscores determined by immunohistochemical analysis, read by two independent observers.

Figure 3. Correlation between PAR-2 histoscores in cancer cells and mRNA levels in ovarian cancers. PAR-2 histoscores and mRNA levels were determined by immunohistochemistry and real-time RT-PCR, respectively. Each level is the mean \( \pm \) SD of nine determinations.

results

PAR-2 localization by immunohistochemistry

Immunohistochemical staining for PAR-2 on a representative case of clear cell carcinoma of the ovary is shown in Fig. 1. The intensity of PAR-2 in stromal cells was extremely lower than...
Figure 4. (A) PAR-2 histoscores and mRNA levels in ovarian cancers classified according to clinical stages. Clinical stages of ovarian cancer were assessed according to FIGO classification. Alive and dead cases are numbered in open circles and closed circles, respectively. Each level is the mean ± SD of nine determinations. *, representative cases from each stage of ovarian cancer are shown in (B) and (C). *P < 0.01; **P < 0.05. (B) Immunohistochemical staining for PAR-2 in ovarian cancers representing each cancer stage (original magnification ×200). (C) The products of RT-PCR for PAR-2 in ovarian cancers representing each cancer stage by 2% agarose gel electrophoresis.
that in the cancer cells in all cases studied, as shown in Fig. 2. Therefore, PAR-2 expression was dominantly distributed in cancer cells rather than in stromal cells. The other PAR-2 antibody showed almost the same localization and intensity of staining. PAR-2 mRNA expression seemed to be supplied mainly from the cancer cells. PAR-2 histoscores in cancer cells correlated with the corresponding mRNA levels in each tissue, as shown in Fig. 3.

levels of PAR-2 according to clinical backgrounds

Both PAR-2 histoscores in cancer cells and mRNA levels in ovarian cancers significantly increased with clinical stages (I < II < III < IV, P < 0.05), as shown in Fig. 4. There was no significant difference in PAR-2 histoscores or in mRNA levels according to histopathological type, as shown in Fig. 5.

correlation of PAR-2 with patients’ overall survival

We divided the patients into two equal groups, as high and low PAR-2 groups, with 24 patients in each group. The cut-off levels were determined by the median values among the 48 patients at a PAR-2 histoscore of 227, and a PAR-2 mRNA level of $1.9 \times 10^4$ DNA copy/µg total RNA. The 36-month survival rate of the 24 patients with high PAR-2 was poor (58%), while that of the other 24 patients with low PAR-2 was significantly better (83%), as shown in Fig. 6.

association of PAR-2 with MVC

PAR-2 histoscores in cancer cells correlated with MVC-CD31 (MVCs determined by immunohistochemistry for CD31; $r = 0.70$, $P < 0.01$) and MVC-FVIII (MVCs determined by immunohistochemistry for factor VIII-related antigen; $r = 0.67$, $P < 0.01$); and PAR-2 mRNA levels also correlated with MVC-CD31 ($r = 0.68$, $P < 0.01$) and MVC-FVIII ($r = 0.78$, $P < 0.01$), as shown in Fig. 7.

discussion

Among other members of PARs, well-described PAR-1 significantly involves in both angiogenesis and tumor invasion and metastasis by activated phosphorylation of focal adhesion complex proteins, cytoskeletal reorganization and αvβ5 integrin recruitment [27]. PAR-1 activation contributes to tumor growth by enhancing tumor cell proliferation as has been shown in melanoma [28] and colon cancers [29]. In breast
cancers, PAR-1 correlates with tumor progression by augmenting the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) [30] and, in prostate cancers, it has been implicated in bone metastasis [31]. Indeed, PAR-1 has been reported to be highly expressed in pancreatic adenocarcinoma [32], laryngeal carcinoma [33], renal carcinoma [34], lung adenocarcinoma, gastric carcinoma [8], and oral squamous cell carcinoma [35]. This is the first study to demonstrate the role of PAR-2 in ovarian cancers, to the best of our knowledge.

In the present study, PAR-2 expressed in the cancer cells increased with tumor advancement of ovarian cancers. The marked difference in survival between patients with high and low PAR-2 in our study suggests that PAR-2 may be a novel indicator of ovarian cancer progression. Microvessel count is an independent significant prognostic factor in patients with breast cancer [36], and both relapse-free and overall survival rates decrease with increasing microvessel count [37]. PAR-2 has been implicated in cellular proliferation, invasion and metastasis, with angiogenesis in cancers of the breast [8, 10], colon [15, 14], gastrrium [12, 13], pancreas [16, 17], lungs [7], prostate [9], and melanoma [9] and glioblastoma [19]. A synergistic effect of PAR-2 with VEGF in alveolar angiogenesis by proliferation of alveolar capillary endothelial cells has been observed in lung adenocarcinoma [7]. In the present study, positive correlation of PAR-2 expression with microvessel counts indicates that PAR-2 may be a candidate for angiogenic mediator as the clinical relevance of angiogenesis is assessed by MVD. Activation of PAR-2 in cancer cells may result in its interacting with vascular endothelial cells, indicating that it might participate in tumor advancement by contributing to angiogenesis.

In summary, this study suggests that PAR-2 promotes the growth of tumor with dissemination of ovarian cancers via angiogenesis, leading to poor survival rates. Thus, inhibition of angiogenesis may not only suppress tumor growth with dissemination, but also improve the prognosis for advanced ovarian cancer patients. It is hoped that further prospective studies of PAR-2 in the advancement of ovarian cancers will provide a novel therapeutic approach in ovarian cancers.

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