Clinical implications of expression of ETS-1 related to angiogenesis in uterine endometrial cancers

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Background: Angiogenesis is essential for development, growth and advancement of solid tumors. During angiogenesis, ETS-1 is strongly expressed in vascular endothelial cells and the adjacent interstitial cells, while the inhibition of ETS-1 expression leads to suppression of angiogenesis. This prompted us to study the clinical implications of ETS-1 in relation to angiogenesis in uterine endometrial cancers.

Patients and methods: Sixty patients underwent resection for uterine endometrial cancers. From the tissues of 60 uterine endometrial cancers, the levels of ets-1 mRNA, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF) and interleukin (IL)-8 were determined by competitive RT–PCR using recombinant RNA and enzyme immunoassay, and the localization and counts of microvessel were determined by immunohistochemistry.

Results: There was a significant correlation between microvessel count and ets-1 gene expression levels in uterine endometrial cancers. Immunohistochemical staining revealed that the localization of ETS-1 was similar to that of vascular endothelial cells. The level of ets-1 mRNA tended to increase with increasing disease stage. Furthermore, the level of ets-1 mRNA correlated with levels of VEGF in well-differentiated adenocarcinomas (G1) and of bFGF in moderately differentiated adenocarcinomas (G2) and poorly differentiated adenocarcinomas (G3).

Conclusions: ETS-1 is a possible angiogenic mediator in uterine endometrial cancers.

Key words: angiogenesis, basic FGF, ets-1, uterine endometrial cancer, VEGF

Introduction

Angiogenesis is essential for development, growth and advancement of solid tumors [1]. The angiogenic factors vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF) identified with thymidine phosphorylase (TP), basic fibroblast growth factor (bFGF) and interleukin (IL)-8 work on angiogenesis in uterine cancers [2–10]. The expression of VEGF, and in particular its VEGF165 and VEGF121 isomers, decreased with advancement of clinical stage and with dedifferentiation in uterine endometrial adenocarcinomas [3]. PD-ECGF expression was significantly higher in well-differentiated adenocarcinomas (G1) than in moderately differentiated adenocarcinomas (G2) and poorly differentiated adenocarcinomas (G3) [7]. Conversely, bFGF expression increased with advancement of clinical stage and with dedifferentiation. VEGF expression in a well-differentiated endometrial cancer cell line was sensitively regulated by ovarian steroids [11], and PD-ECGF expression in uterine endometrium was also sensitively regulated by ovarian steroids [12]. These results indicate that VEGF and PD-ECGF expressions might be down-regulated with dedifferentiation. Therefore, if VEGF and PD-ECGF expression in G1, and bFGF expression in G2 and G3 can be suppressed by the use of tumor dormancy therapy, patient prognosis should be improved remarkably without the severe side effects seen with chemotherapy. Because the effects of chemotherapy are not specific to cancer cells, it can produce severe side-effects in normal cells, especially bone marrow cells. On the other hand, tumor dormancy therapy is specific to the rapidly growing vascular endothelial cells in tumors, and has no effect on slow-growing vascular endothelial cells or other normal cells. However, if an angiogenic factor is suppressed by tumor dormancy therapy over a long period of time, another angiogenic factor might be induced by an alternately linked angiogenic pathway, a process referred to as tolerance.

During angiogenesis, ETS-1 is strongly expressed in vascular endothelial cells and the adjacent interstitial cells...
and synthesized by PCR from a Deoxynucleic acid. The internal standard was originated for competitive RT–PCR and Southern blot analysis [21].

Preparation of internal standard recombinant RNA (rcRNA) for competitive RT–PCR and Southern blot analysis [21]

Deoxynuclease construction of the internal standard was originated by PCR from a BamHI/EcoRI fragment of V-erbB (Clontech, Palo Alto, CA, USA) with two sets of oligonucleotide primer sequences. The sequences for the first set of primers for ets-1 mRNA (MIMIC ets-1-5′ and MIMIC ets-1-3′) in the first PCR were as follows: MIMIC ets-1-5′, 5′-ATGGAGTCAACCCAGCTCTGCAAGTGA-ATCTCTCTCCG-3′; MIMIC ets-1-3′, 5′-CCATGCACTAGTGTCTGTCGGTCTGCGTAG-3′ [22, 23]. The sequences for the second set of primers for ets-1 mRNA (MIMIC ets-1-5 and ets-1-3′) in the secondary PCR were as follows: MIMIC ets-1-5′, 5′-TAATACCGACTCCTATAGGATGGAGTCAACCCAGCTCTGCAAGTGA-ATCTCTCTCCG-3′; MIMIC ets-1-3′, 5′-CAGCTAGTGTCTGTCGGTCTGCGTAG-3′. The first and second PCRs were carried out as previously described [21]. The second PCR products were transcribed using T7 RNA polymerase (Gibco BRL, Gaithersburg, MD, USA) and the amount of transcribed internal marker was calculated as previously described [21].

Figure 1. Quantitative analysis of ets-1 mRNA by competitive RT–PCR and Southern blot analysis. RT–PCR reactions containing ets-1 gene-specific primers were carried out in the presence of total RNA and serial diluted internal standard recombinant RNA (rcRNA) in the range of 1–100 fmol for ets-1 mRNA. (A) Southern blot analysis for competitive RT–PCR. (B) Data are plotted to determine ets-1 mRNA levels as the log ratio of rcRNA/ets-1 mRNA total RNA isolated from the samples versus log rcRNA.

Competitive RT–PCR and Southern blot analysis

Total RNA was isolated from tissues by the acid guanidium thiocyanate–phenol–chloroform extraction method [24]. To obtain a standard curve for ets-1 gene-specific primers, a dilution of recombinant RNA for ets-1 mRNA (1–100 fmol) were reverse transcribed. Primer sequences used to amplify the ets-1 gene (ets-1-5′ and ets-1-3′) were as follows: ets-1-5′, 5′-ATGGAGTCAACCCAGCTCTGCAAGTGA-ATCTCTCTCCG-3′; ets-1-3′, 5′-CCATGCACTAGTGTCTGTCGGTCTGCGTAG-3′. Competitive PCR was carried out as previously described [21]. In the competitive RT–PCR and Southern blot analysis for ets-1 mRNA, only two predicted sizes of DNA fragment were hybridized with the biotinylated ets-1-5′ probe to determine quantity as previously described [21]. As a negative control, no ets-1 mRNA was detected without reverse transcription in 30 cycles of PCR. Levels of ets-1 mRNA were determined using a standard curve and a serial dilution of rcRNA in competitive RT–PCR and Southern blot analyses, as shown in Figure 1.

Immunohistochemistry

Sections (4 μm) of formalin-fixed paraffin-embedded tissues of uterine endometrial cancers were cut with a microtome and deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. The samples for ETS-1 were soaked in citrate buffer and then microwaved at 100°C for 10 min, and those for factor VIII-related antigen 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 ETS-1 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-factor VIII-related antigen (Zymed, San Francisco, CA, USA) were used at dilutions of 1:2000 and 1:2, respectively, as the first antibodies. The addition of the first antibody, rabbit anti-human ETS-1 or rabbit anti-factor VIII-related antigen, was omitted in the protocols for negative controls of ETS-1 or factor VIII-related antigen, respectively.

Vessels were counted in the five highest density areas at 200× magnification (using a combination of 20× objective and 10× ocular, 0.785 mm² per field). Microvessel counts were expressed as the mean numbers of vessels in these areas [25]. Microvessel density was evaluated by the counting of microvessels.

Enzyme immunoassay for determination of bFGF, VEGF, PD-ECGF and IL-8 antigens

All steps were carried out at 4°C. Uterine endometrial cancers tissue (wet weight 10–20 mg) was homogenized in HG buffer (5 mM Tris–HCl pH 7.4, 5 mM NaCl, 1 mM CaCl₂, 2 mM ethyleneglycol-bis-[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid, 1 mM MgCl₂, 2 mM DTT, 25 µg/ml aprozin and 25 µg/ml leupepint) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12 000 r.p.m. (10 000 g) for 3 min to obtain the supernatant. The protein concentration of samples was measured by the method of Bradford [26] to standardize VEGF, bFGF, PD-ECGF and IL-8 antigen levels.

Basic FGF, VEGF and IL-8 antigen levels in the samples were determined by a sandwich enzyme immunoassay using Human bFGF, VEGF and IL-8 Quantikine kits (R&D System, Minneapolis, MN, USA), respectively. PD-ECGF antigen levels were determined by the method of Nishida et al. [27]. The levels of bFGF, VEGF, PD-ECGF and IL-8 were standardized with the corresponding cellular protein concentrations.

Statistics

Levels of ets-1 mRNA, VEGF, bFGF, PD-ECGF and IL-8 were measured from three parts taken from each tissue, and the assay for each sample was carried out in triplicate. The t-test for two independent samples was used to compare the determinations in Figure 4. The sample correlation coefficient was used for the comparisons in Figures 3, 5 and 6. Differences were considered significant for values of $P < 0.05$.

Results

Immunohistochemical staining for ETS-1 and factor VIII-related antigen in a representative case of G2, stage IIb is shown in Figure 2. Factor VIII-related antigen was clearly distributed in vascular endothelial cells. ETS-1 was also distributed in vascular endothelial cells and in the adjacent interstitium. There was a significant correlation between microvessel counts (MVC) and ets-1 mRNA levels ($P < 0.001$) in uterine endometrial cancers (Figure 3).

Ets-1 mRNA levels tended to increase with increasing disease stage of uterine endometrial cancers (Figure 4). There was a significant correlation between ets-1 mRNA and VEGF ($P < 0.001$) in G1 (Figure 5), but not between ets-1 mRNA and bFGF, PD-ECGF or IL-8 (data not shown). There was a significant correlation between ets-1 mRNA and bFGF...
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**Discussion**

Ets-1 is expressed in a variety of cancer cells including gastric, pancreatic, esophageal, hepatocellular and cholangiocellular carcinomas, and thyroid and astrocytic tumors, and acts as a proto-oncogene during tumor progression [28–34]. ETS-1 is up-regulated and is involved in the overexpression of MMP-7 in hepatocellular carcinoma cells [35], and positively regulates the expression of uPA in breast cancer, glioma, astrocytoma and meningioma cells, related to invasive potential and phenotypes [36–39]. ETS-1 expression is induced by bFGF in glioma cells, related to invasive potential [37], and by VEGF in astrocytoma, related to angiogenesis [40]. Furthermore, overexpressed ETS-1 is recognized as an angiogenic mediator in oral squamous cell carcinomas and gastric carcinomas [41, 42].

In the current study, transcription factor ETS-1 was dominantly expressed in vascular endothelial cells and their adjacent interstitium, but not in cancer cells in uterine endometrial cancers, while ets-1 mRNA levels correlated with microvessel density observed in immunohistochemical staining for factor VIII-related antigen. Generally, distinct ETS-1 expression in vascular endothelial cells has been recognized as evidence of accelerated angiogenesis [13–15]. The present data reveal that ets-1 mRNA levels increased with increasing disease stage in uterine endometrial cancers. Therefore, ETS-1 might be activated as a direct angiogenic mediator for the initiation and maintenance stages of angiogenesis, and may possibly be an excellent indicator of patient prognosis in uterine endometrial cancers.

Since levels of ets-1 mRNA in this study correlated with levels of VEGF in G1 and bFGF in G2 and G3, it can be concluded that in all cases VEGF and bFGF act as angiogenic factors in uterine endometrial cancers, especially VEGF in G1 and bFGF in G2 and G3. We previously reported that VEGF expression was down-regulated with dedifferentiation (G1→G2→G3) [3] and conversely bFGF expression was up-regulated with dedifferentiation in uterine endometrial cancers [9]. This indicates that ETS-1 is an angiogenic mediator linked to VEGF in G1 and bFGF in G2 and G3, and preserves angiogenic switching in the linkage to angiogenic factors to maintain advancement. Also, it is well known that bFGF and VEGF induce ETS-1 expression in vascular endothelial cell lines [16, 17]. Therefore, even if VEGF or bFGF can be suppressed by some agents, angiogenesis might be suppressed only transiently, which could lead to a temporary suppression of tumor growth and secondary spreading. In such a scenario,
Figure 5. Correlation between ets-1 mRNA and VEGF levels in uterine endometrial cancers. Each level is the mean of nine determinations.

Figure 6. Correlation between ets-1 mRNA and bFGF levels in uterine endometrial cancers. Each level is the mean of nine determinations.
other angiogenic factors would be induced, and link to ETS-1 in the recruitment for alternate angiogenic activation, as a kind of tolerance to angiogenic factors. Therefore, suppression of the major angiogenic factors along with suppression of ETS-1 recruitment might be more effective as a tumor dormancy therapy than mere suppression of major angiogenic factors. A specific inhibitor for ETS-1, transdominant mutant ETS-1, has already been shown to act as a dominant negative molecule and can be used as an efficient tool for angiogenic inhibition [43].

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