Small Interfering RNA Molecules Targeting Endothelin-Converting Enzyme-1 Inhibit Endothelin-1 Synthesis and the Invasive Phenotype of Ovarian Carcinoma Cells

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Abstract

Endothelin-1 (ET-1) has been implicated in the progression of various cancers, including ovarian carcinoma. We found that the ovarian carcinoma cell lines ES2 and OVCA3 and tumors from different anatomic sites expressed ET-1 system members [ET receptor A and ET-converting enzyme-1 (ECE-1)]. However, only ECE-1 was significantly higher in the solid tumors compared with effusions. We therefore investigated the effect of RNA interference-induced knockdown of ECE-1, the key enzyme in ET-1 production, on these two ovarian carcinoma cell lines. Small interfering RNA (siRNA) targeting of ECE-1 markedly reduced ECE-1 mRNA and protein levels, which subsequently led to 80% to 90% inhibition of ET-1 peptide secretion by the cells. ECE-1 silencing also profoundly affected the behavior of tumor cells compared with cells treated with scrambled siRNA. Silenced cells exhibited (a) reduced ET-1–dependent p44/42 mitogen-activated protein kinase phosphorylation; (b) decreased invasiveness and matrix metalloproteinase-2 activity; (c) improved adhesion to basal lamina proteins, laminin-1, and collagen IV; and (d) increased E-cadherin, an epithelial adhesion molecule, and reduced N-cadherin expression, a mesenchymal marker. Altered cell adherence is one of the hallmarks of the transformed phenotype, often characterized by the loss of the epithelial features and the gain of a mesenchymal phenotype. ECE-1 ablation did not, however, alter viable ovarian carcinoma cell numbers. Addition of exogenous ET-1 reversed the effects cited above. Taken together, these data indicate that siRNA is an effective tool for manipulating ECE-1 expression, ET-1 biosynthesis, and invasiveness of ovarian carcinoma. ECE-1 silencing may therefore develop into a promising novel anticancer therapy. [Cancer Res 2008;68(22):9265–73]

Introduction

Ovarian cancer, the leading cause of death from gynecologic malignancy, is a highly metastatic disease (1). Both the incidence and death rate from this tumor seem to be on the rise in Western countries (2). Endothelin-1 (ET-1) was shown to be a key regulator of normal ovarian function (3) and of ovarian carcinoma tumor progression (4). ET-1 is a pleiotropic peptide that is involved in numerous physiologic and pathologic processes (5–7). Its multiple effects are mediated by G protein–coupled receptors, termed ETA and ETB (8, 9). Coupling of activated ET receptors to multiple G proteins may explain the involvement of ET-1 in diverse cellular processes (5–7). Most primary and metastatic ovarian cancers express ET-1 and ETA receptor mRNA as well as ET-1 peptide (10). ET-1 expression has also been reported in other human malignancies (11–13).

Decreased intercellular adhesiveness is one of the most common alterations in human cancers (14–16). The cadherin superfamily of Ca²⁺–dependent homophilic adhesion molecules regulates cell-to-cell interactions (14). E-cadherin, a member of the cadherin family, is essential for maintaining adherens junctions, which confer physical integrity and polarization of epithelial cells. Cell invasion is also facilitated by matrix metalloproteinases (MMP), a family of zinc- and calcium-dependent enzymes, which degrade extracellular matrix components (17, 18). Both processes were reported to be affected by ET-1 (19, 20). Activation of ETA was also shown to promote ovarian cancer tumor progression, proliferation, survival, and angiogenesis (4, 21). Therefore, ETA antagonists were suggested as a potential anticancer therapy.

ET-1 transcript is initially translated as a preproprotein of 200 amino acids; its proteolytic cleavage generates the inactive precursor big ET-1. Big ET-1 requires cleavage to an active form, carried out by ET-converting enzyme-1 (ECE-1), a zinc-binding metalloendopeptidase (22). ECE-1–deficient mice display various defects in cephalic, cardiac, vagal, and neural crest structures, reproducing the combined phenotype of ET-1–deficient or ETA/ETB-deficient mice (7, 23), thus showing the physiologic significance of ECE-1 in generating ET-1. In recent years, other pathways for big ET-1 processing have been proposed (24). Because ET-1 is a short, nonglycosylated peptide, its half-life is very short. Therefore, the final step in synthesizing ECE-1 must take place in proximity to its site of action, which may explain why its expression is widespread even in cells that do not express ETs. ECE-1 is found in endothelial cells throughout the body, but it is also highly expressed in other cell types, such as testicular Leydig cells, adrenal cells, and ovarian granulosa cells (25, 26). Four full-length, membrane-bound isoforms of ECE-1 (1a, 1b, 1c, and 1d), which only differ in their 5’ end, are known and have been characterized (27, 28). Recently, we reported a splice variant lacking the transmembrane domain of the enzyme (29). These various ECE-1 isoforms all contain the catalytic site (30) and thus share similar enzymatic activities; however, they differ in their cellular and subcellular localization (30–32).
To date, the role of ECE-1 in ovarian cancer has not been studied, and open questions remain unanswered, such as do ovarian carcinomas express ECE-1, and how is ECE-1 relevant to ET-1 secretion and to ovarian cancer cell behavior? We found that ECE-1 is elevated in solid ovarian cancer tumors, compared with effusion, and that the knockdown of ECE-1 using small interfering RNA (siRNA) is an efficient method to suppress ET-1 secretion and to reduce the invasive phenotype of the cells.

Materials and Methods

Cell culture and transfection. Human ovarian carcinoma cell lines OVCAR3 (HTB-161) and ES2 (CRL1978) were from the American Type Culture Collection. The cells were grown in complete DMEM (high glucose) containing 10% FBS, 1% glutamine, 1% sodium pyruvate, 1% vitamins, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin (Biological Industries) in a humidified atmosphere of 95% air and 5% CO2. OVCAR3 and ES2 cells were seeded at 100 × 10^3 in six-well tissue culture plates and transfected the next day with various concentrations of siRNA duplexes targeting ECE-1 sequences common to all forms [sense, rAGAUCAUC-CUGGAGAUUAA]dTdT or with scrambled siRNA designated a negative control (Sigma-Prologol) as indicated in the text. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After transfection, the culture medium was replaced daily and the cells were harvested at the times indicated in the text.

For the phosphorylation experiments, ECE-1–silenced cells and negative controls were serum deprived for 24 h. Then, cells were incubated with basal medium (without FBS) or medium containing 100 mmol/L ET-1 or 40 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) for 10 min. MAPK phosphorylation, adhesion, basement membrane invasiveness, and MMP assays were carried out 4 to 5 d after transfection.

Clinical specimens. All specimens were obtained from the Pathology Clinic at the Norwegian Radium Hospital. The Regional Committee for Medical Research Ethics in Norway approved the study. The analyzed material consisted of 14 primary carcinomas, 16 effusions, and 17 solid metastases (total, 47). Effusions were centrifuged and frozen as cell pellets immediately on arrival. Solid tumor specimens were similarly immediately snap-frozen in liquid nitrogen. Frozen sections were obtained from all solid tumors to verify the presence of sufficient (>50%) tumor and the absence of necrosis.

RNA extraction and real-time PCR. Total RNA was extracted from the cells using PeqGOLD TriFast reagent (Peviva) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a total volume of 20 μL using 200 units reverse transcriptase, 50 pmol random hexamer, and 0.075 nmol oligo(dT). Real-time PCRs were performed using the Stratagene Mx3000P quantitative PCR system, using the SYBR Green I PCR kit with ROX passive reference (Eurogentec). Standard curves of cDNA samples were generated for each set of primers using serial dilutions. In the initial experiments, the expected PCR products were verified by agarose gel electrophoresis and DNA sequencing. Each real-time reaction (18 μL) contained SYBR Green Master Mix (200 μmol/L deoxynucleotide triphosphates, 5 mmol/L MgCl2, uracil-N-glycosylase, and AmpliTaq HotGoldStar DNA polymerase), 0.54 μL of a 1:10,000 dilution of SYBR Green stock solution, 1.5 mmol/L deoxynucleotide triphosphates, 500 nmol/L of each primer (a list of real-time PCR primers is shown below), and 25 to 50 ng cDNA. The reactions were performed in 0.2 μL PCR tubes capped with Micro-Amp optical caps. The reactions were incubated at 95°C for 10 min to activate the AmpliTaq Gold polymerase followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for both annealing and elongation. Dissociation curve analysis was performed after each real-time experiment to confirm the presence of only one product and the absence of primer dimers. The relative expression of the reverse transcription-PCR (RT-PCR) products was determined using the ΔCt method (Livak and Schmittgen, 2001). This method calculates the relative expression using the equation fold induction = 2−[ΔCt], where Ct = the threshold cycle (i.e., the cycle number at which the relative fluorescence of the sample increases above the background fluorescence) and ΔCt = [Ctgene of interest – CtG3PDH]. Each sample was run in duplicate and the mean Ct was used in the ΔCt equation. Primers used were the following: G3PDH, GGAGAACTGTGGCTGTAGT and CGTTGACGCTCAGGAGATGC; ECE-1, GGAGAATCGCCGACAAC and TGCAAAACCCAGGAAGAAG; ET-1, CTGCCACCGACATCATTTG and TCTCAGGTCGTTGCGCTTTG; and ETA, GTCTCTGTCTGATCACTACGAA and GTGCTGTCGGGCAA-TAGTGG.

Western blot analysis. Cells were washed thrice with PBS and proteins were extracted by adding sample buffer [0.5 mol/L Tris-HCl (pH 6.8), 25% (v/v) glycerol, 10% (v/v) SDS, 0.5% (v/v) bromphenol blue, 5% (v/v) β-mercaptoethanol]. All steps were performed on ice, and samples were kept frozen until use. The samples were separated by 12.5% SDS-PAGE under reducing conditions. Proteins were electrically transferred to nitrocelulose membranes. After having been blocked for 2 h in TBST (20 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween 20) containing 5% low-fat milk,
the membranes were incubated with the respective antibodies: anti-hECE-1 (antiserum 29, directed against the catalytic domain; diluted 1:10,000), polyclonal N-cadherin and E-cadherin (each diluted 1:800; Santa Cruz Biotechnology), p44/42 total MAPK (diluted 1:50,000), and p44/42 phosphorylated MAPK (diluted 1:1,000; Cell Signaling). Antibodies were diluted in 5% bovine serum albumin (BSA) and incubated for 2 h at room temperature. The membranes were then washed thrice with TBST (10 min) and incubated with peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature. A chemiluminescent signal was generated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and the Western blots were exposed to X-ray films and subsequently scanned as previously described (33, 34).

ELISA. ET-1 concentrations in culture medium were determined directly (without extraction) by using the ELISA kit (Cayman Chemical) according to the manufacturer's instructions. For determination of ET-1 concentrations, cells were cultured in serum-free medium for 6 h before collection. Medium was then centrifuged and stored at −20°C. ET-1 was detected in the range of 1.5 to 250 pg/mL.

Adhesion. Ninety-six–well plates used for attachment assays were coated with various concentrations of laminin-1 and collagen IV and were blocked by 2% BSA. Cells were harvested by brief exposure to 1 mmol/L EDTA, washed with DMEM containing 0.1% BSA, and then added to the matrix-coated wells (200,000 cells). The chambers were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air for 6 h. Next, the cells traversed the Matrigel layer and were attached to the lower surface of the filter. Finally, the cells were stained with DiffQuik (Dade Diagnostics) and counted in five random fields.

Determination of MMP activity (zymography). Subconfluent cell cultures were incubated for 24 h in serum-free DMEM and the resulting supernatant was analyzed for collagenolytic activity. The collagenolytic activity was determined on a gelatin impregnated (1 mg/mL; Difco), 8% SDS-PAGE gel, as previously described (37).

Statistical analysis. Data are presented as means ± SE. Student’s t test was used to evaluate differences between groups. For multiple comparisons, the one-way ANOVA Tukey-Kramer test was used to determine the statistical difference between the groups. To identify the differences between ovarian cancer specimens, statistical analysis was performed with nonparametric test methods using the Kruskal-Wallis H test. Differences were considered significant at P < 0.05.

Results

Expression of endothelin system genes at different anatomic sites in ovarian carcinoma. Initially, we quantified ET-1, ECE-1, and ETA mRNA expression in specimens from ovarian carcinoma patients taken from different anatomic sites. ET-1, ECE-1, and ETA

The membranes were incubated with the respective antibodies: anti-hECE-1 (antiserum 29, directed against the catalytic domain; diluted 1:10,000), polyclonal N-cadherin and E-cadherin (each diluted 1:800; Santa Cruz Biotechnology), p44/42 total MAPK (diluted 1:50,000), and p44/42 phosphorylated MAPK (diluted 1:1,000; Cell Signaling). Antibodies were diluted in 5% bovine serum albumin (BSA) and incubated for 2 h at room temperature. The membranes were then washed thrice with TBST (10 min) and incubated with peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature. A chemiluminescent signal was generated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and the Western blots were exposed to X-ray films and subsequently scanned as previously described (33, 34).

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Determination of basement membrane invasiveness. Boyden chamber chemoinvasion assays were performed as previously described (35, 36). Matrigel (reconstituted basement membrane; 25 μg) was dried on a polycarbonate filter (Nucleopore polyester PVP free, Whatman International Ltd.). Fibroblast-conditioned medium (obtained from confluent NIH-3T3 cells cultured in serum-free DMEM) was used as the chemotactic attractant. Cells were harvested by brief exposure to 1 mmol/L EDTA, washed with DMEM containing 0.1% BSA, and then added to the Boyden chambers (200,000 cells). The chambers were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air for 6 h. Next, the cells traversed the Matrigel layer and were attached to the lower surface of the filter. Finally, the cells were stained with DiffQuik (Dade Diagnostics) and counted in five random fields.

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mRNA were detected in samples from primary ovarian tumors (n = 14), metastases (n = 17), and effusions (n = 16). Whereas ET-1 and ETA mRNA levels were not significantly different among these sites, ECE-1 levels were significantly higher in the solid tumors (primary and metastases) than in effusions (mean rank = 26, 29, and 16 for primary carcinomas, solid metastases, and effusions, respectively; P = 0.021). Median levels for tumors at these three anatomic sites were 2.76, 6.28, and 1.56, respectively (Fig. 1).

Characterization of endothelin system genes in ovarian carcinoma cell lines. To further study the role of ET-1 in ovarian cancer, we chose two cell lines of different histologic type: clear cell (ES2) and serous (OVCAR3). ET-1, ETA, and ECE-1 expression were determined in these ovarian cancer cell lines. ET-1, ETA, and ECE-1 were all expressed in these cell lines, although at different levels (Fig. 2). ET-1 mRNA expression was considerably higher in ES2 cells compared with OVCAR3 cells (Fig. 2A). Interestingly, however, secreted ET-1 peptide concentrations were similar in the two cell types (Fig. 2B), most probably as a result of the higher ECE-1 present in OVCAR3 cells (Fig. 2C). This finding emphasizes the significance of ECE-1 in ET-1 production. The

Figure 3. Suppression of ET-1 biosynthesis by ECE-1 siRNA molecules. A and B, dose-response effect of ECE-1 siRNA molecules on ECE-1 silencing in the ES2 and OVCAR3 cell lines, respectively. ECE-1 mRNA levels were determined by real-time PCR 3 and 6 d after transfection. Results are presented as the percent inhibition relative to cells treated with scrambled siRNA (negative control; designated 100%). Mean ECE-1 mRNA levels were calculated using the ΔCt method (see Materials and Methods). Different letters indicate significant differences among siRNA doses (P < 0.05). C, ECE-1 protein expression. The ES2 and OVCAR3 cells were treated with 25 nmol/L of ECE-1 siRNA or scrambled siRNA (negative control). Cells were extracted 6 d after transfection and analyzed by Western blotting using a specific antibody directed against the catalytic domain of ECE-1. The membranes were also probed with anti-total MAPK (p44/42) antibody to correct for protein loading. D, effect of ECE-1 siRNA molecules on ET-1 secretion into the culture medium. The ES2 and OVCAR3 cells were treated with 25 nmol/L of ECE-1 siRNA or scrambled siRNA as a negative control. Three, 5, and 6 d after transfection, the culture medium was collected and ET-1 concentrations were determined by ELISA. The results are depicted as the percent of ET-1 peptide secreted in ECE-1–silenced ES2 (○) and OVCAR3 (●) compared with peptide secreted from cells treated with scrambled siRNA used as a negative control (designated 100%).
levels of ETA mRNA (Fig. 2D) were also higher in OVCAR3 cells than in ES2 cells.

ECE-1 RNA interference knockdown. These cell lines were then used to determine whether manipulation of the ECE-1 gene could affect ET-1 production by ovarian carcinoma. Preliminary experiments were conducted to examine siRNA transfection efficiency. For that purpose, cells were transfected with different concentrations of FITC-labeled siRNA and labeled cells were analyzed by fluorescence-activated cell sorting. At least 85% of the cells were labeled using 10 to 25 nmol/L of siRNA (data not shown). ES2 and OVCAR3 were then transfected with varying concentrations (5, 10, 25, and 50 nmol/L) of siRNA, targeting a common region of ECE-1 isoforms or with scramble siRNA serving as a negative control. ECE-1 mRNA was measured 3 and 6 days after transfection. As shown in Fig. 3, at 3 days after transfection, 10 nmol/L were sufficient to maximally inhibit ECE-1 mRNA in both cell types (84% and 93% inhibition in ES2 and OVCAR3, respectively). In fact, in OVCAR3, even 5 nmol/L gave maximal inhibition. The inhibition persisted for at least 6 days, although to a lesser degree. For the next experiments, a dose of 25 nmol/L siRNA was used. To determine whether the ECE-1 inhibition was also evident at the protein level, we measured the expression of ECE-1 by immunoblotting using anti-hECE-1 antibody directed against the common catalytic domain (Fig. 3C). Six days after ECE-1 siRNA transfection, only a small fraction of ECE-1 protein remained compared with the negative control in both cell lines (13% and 36% for OVCAR3 and ES2, respectively; Fig. 3C). Levels of ECE-1 protein expression 3 days after transfection showed even stronger inhibition (data not shown).

It was important thereafter to determine whether ECE-1 knockdown affected ET-1 secretion. The data in Fig. 3D show that the amounts of ET-1 peptide secreted by the silenced cells nicely corresponded to ECE-1 silencing (mRNA and protein). On days 3 and 5, there was 80% to 90% inhibition in OVCAR3 and ES2 cells, respectively. A marked inhibition (>70%) was also observed on day 6 in OVCAR3 cells, whereas in ES2 cells the inhibition at this stage was smaller (Fig. 3D).

Effect of ECE-1 silencing on the ovarian cancer cell phenotype. ECE-1 silencing reduces ET-1–dependent p44/42 MAPK phosphorylation (Fig. 4A). In fact, in ECE-1–silenced OVCAR3 cells, the phosphorylation of p44/42 MAPK was almost completely abrogated. In contrast, TPA, which activated the MAPK pathway via protein kinase C activation, strongly stimulated p44/42 MAPK phosphorylation in both cells transfected with scramble siRNA and ECE-1–targeted siRNA (Fig. 4A). ETA mRNA was also measured, revealing a significant reduction following ECE-1 knockdown (Fig. 4B). This suggests that reduced receptor levels may have contributed to ET-1–dependent p44/42 MAPK phosphorylation.

The ability of the ECE-1–silenced cells to invade the basement membrane was then examined using the Boyden chamber chemoinvasion assay. In both cell lines, there was a 2-fold decrease in invasiveness of the silenced cells compared with the negative control cells. Moreover, decreased invasiveness in ECE-1 knockdown cells was reversed by adding back 200 pg/mL ET-1 to the culture medium (Fig. 5A). In contrast, adding ET-1 to cells transfected with negative control siRNA did not significantly affect the invasiveness of the cells. Gelatin zymography revealed a 2-fold decrease in MMP-2 activity in the silenced cells (Fig. 5B), a phenomenon that may be involved in the decreased invasiveness exhibited by ECE-1–silenced cells.

We next examined the ability of ECE-1 siRNA–treated ovarian cancer cell lines to adhere to culture wells coated with two proteins that are an integral part of the structural scaffolding of basement membranes: laminin-1 and collagen IV. Attachment assay results (Fig. 6A and B) showed a significant increase in the adhesion of ECE-1 siRNA–treated cells to both laminin-1 and collagen IV compared with scrambled siRNA–treated cells. Adhesion was higher with collagen IV than with laminin-1 in both the ES2 and OVCAR3 cell lines. As with the invasiveness, adding exogenous ET-1 counteracted the effect of ECE-1 siRNA on cell adhesion, which returned to levels exhibited by the controls (Fig. 6A and B).

The data presented in Fig. 6C show that ECE-1 knockdown also modulated cadherin expression in ovarian cancer lines. There was more than a 3-fold increase in the epithelial cell marker E-cadherin in ECE-1 siRNA–treated OVCAR3 cells and more than a 5-fold decrease in the mesenchymal marker N-cadherin in ECE-1–silenced ES2 cells compared with their respective negative controls (Fig. 6D).

Discussion

Ovarian cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecologic malignancy, and the second most commonly diagnosed gynecologic malignancy (1, 2). Epithelial ovarian cancer is a heterogeneous group of neoplasms that can be divided into different histologic subgroups, each with its own underlying molecular genetic events (38–40).

The data reported here show that siRNA targeting ECE-1 successfully suppressed ECE-1 expression and ET-1 biosynthesis in ovarian cancer cell lines. Moreover, ET-1 knockdown was accompanied by reduced tumorigenic features of the cells, such as...
invasiveness, basement membrane adhesion, and expression of adhesion molecules. ET-1 signaling was also reduced, which was proven in two different ovarian cancer cell lines: ES2 and OVCAR3.

In 1994, ECE-1 was identified as the protease responsible for catalyzing the conversion of 38-amino acid inactive big ET-1 precursor into ET-1 (22), but since then, questions have been raised as to its significance in ET-1 biosynthesis. The excellent correlation between ECE-1 and ET-1 peptide secretion, under basal conditions and after the inhibition of ECE-1 expression, strongly supports the key role of ECE-1 in generating mature and active peptide. The reversal of ECE-1 knockdown by exogenous ET-1 further supports this notion.

Metastases to serosal surfaces and associated peritoneal and/or pleural effusions are found in most patients diagnosed with epithelial ovarian or primary peritoneal carcinoma (41, 42). This is most prominent in the serous or clear cell type histologic types that are known for their aggressive behavior (43). Studies in which effusions and the corresponding solid tumors were compared showed that cells in effusions are different from their counterparts in solid primary and metastatic lesions (40, 42). By examining the expression of ECE-1 in solid tumors and in effusion samples, we found that it was significantly higher in solid tumors than in effusions, whereas ET-1 and ETA did not differ significantly between samples. Peptide content in these samples could not be measured, but based on the arguments above, it can be postulated that elevated ECE-1 levels are sufficient to increase ET-1 secretion even in the absence of elevated ET-1 gene expression. The clinical significance of these findings is not yet fully understood, but it may suggest that the solid tumors are more dependent on ET-1 than effusions. Note that reports dealing with the role of ET-1 in ovarian carcinoma often state that ET-1 expression is elevated in cancerous tissue compared with normal tissue (4, 44). However, the entire normal ovary is not an adequate control tissue, first, because most (>90%) of the ovarian cancers arise from the surface epithelium, and not from the ovarian stroma, which constitutes almost the entire ovarian tissue and, second, because even in ovarian structures, such as follicles and corpus luteum, ET-1 was shown to fluctuate during the reproductive cycle (3). Therefore, the question whether ET-1 or ECE-1 or both are elevated in cancerous versus normal surface epithelium remains unresolved.

![Figure 5](image_url)

**Figure 5.** Effect of ECE-1 silencing on cell invasiveness and MMP activity in ovarian cancer cell lines. ES2 and OVCAR3 cells were treated with 25 nmol/L of scrambled siRNA (Negative Control) or ECE-1 siRNA (siRNA hECE-1) in the presence or absence of 200 pg/mL ET-1 in the culture medium. A, the invasion ability of the cells was measured using a Boyden chamber invasion assay. B, gelatin zymography showing the activity of MMP-2. Representative zymogram for each ES2 and OVCAR3 is shown. C, quantification of MMP-2 activity in three independent experiments (percent change from negative control designated 100%). *, P < 0.05; **, P < 0.01.
The ET-1/ETA autocrine pathway has been implicated in the progression of ovarian carcinoma, and extracellular signal-regulated kinase (ERK) phosphorylation is one of the main signaling molecules activated in this process (45). Importantly, we observed a marked reduction in ET-1–induced ERK phosphorylation following ECE-1 siRNA treatment. This may have been caused by reduced ETA mRNA expression detected in the silenced cells but also by reduced signaling molecules upstream of ERK. In either case, these results suggest that the ETA pathway in ovarian cancer cell lines is sensitized by ET-1. Such homologous up-regulation may be instrumental in the ability of ET-1 to control tumor growth.

Previous reports indicated that ET-1 induced the activity of the MMP—metastasis-related family of proteinases (20). Our study is in accordance with these studies because ECE-1–silenced cells exhibited reduced cell invasiveness and reduced MMP-2 activity, which is partly responsible for the reduced invasiveness. Interestingly, no similar inhibition could be detected in MMP-9 activity (data not shown). Additionally, these cells exhibited increased adhesion to the basement membrane proteins, laminin and collagen type IV, most likely as a result of altered integrin activity. Furthermore, ECE-1 silencing reversed the typical E-cadherin (OVCAR3) and N-cadherin (ES2) expression exhibited by the nonsilenced cells. In ovarian cancer cells (46) and in other types, there is a coordinated down-regulation of both integrins and cadherins (47), showing increased cell-cell and cell-matrix adhesion in the ECE-1–silenced cells, which reduce the ability of the cells to migrate. Addition of exogenous ET-1, in amounts that mimic endogenously secreted peptide, reversed the effects induced by siECE-1, showing the specificity of this treatment. Reduced invasiveness, increased matrix adhesion, and cell-cell interactions, which took place concurrently with ECE-1 (and ET-1) knockdown, support and extend previous studies showing the role of ET-1.
in cancer metastasis (4). Defective cell adherence to the extracellular matrix is a part of acquiring a metastatic phenotype, as is the loss of the epithelial features and a gain of a mesenchymal phenotype (48).

In contrast, ECE-1 knockdown did not alter viable cell numbers (see Supplementary Fig. S1), thus arguing against a role for ET-1 in ovarian carcinoma cell proliferation and/or survival (44). Although this finding is in line with several publications (12, 49), it contrasts some reports in ovarian carcinoma (50). A plausible explanation for this discrepancy lies in the different experimental design of these studies. Here, the effects produced by endogenously secreted ET-1 were shown (Figs. 5 and 6), whereas in many other studies (20, 45, 51), exogenous ET-1 was applied in high concentrations (100 nmol/L versus endogenous release in the order of picomolar amounts), which may exceed the autocrine effect of ET-1 in ovarian carcinoma.

Another prominent aspect of tumor progression is angiogenesis. Here too, ET-1 may play a role. Acting via ETB receptors present on endothelial cells, ET-1 modulates various stages of neovascularization, including endothelial cell proliferation, migration, and invasion (52, 53). ECE-1 knockdown is therefore a promising approach for inhibiting ET-1 release as well as for inhibiting its actions in endothelial cells. Previously, selective ETA antagonists and green tea extracts were proposed as therapeutic tools in ovarian carcinoma (50, 54). ECE-1 siRNA knockdown constitutes another approach able to simultaneously target cancer and endothelial cells, therefore affecting the malignant process and the angiogenesis that accompanies it.

Moreover, ET-1 has been implicated in numerous other human malignancies, including prostate (13), breast (11), and colorectal cancer (12). Therefore, ECE-1 knockdown can also be applied to these other tumors. Interestingly, it was reported that breast cancer patients with ECE-1–overexpressing tumors had more frequent disease recurrence and that ECE-1 inhibition, using an enzyme inhibitor, in MCF-7 breast cancer cells led to significantly decreased ET-1 expression and reduced cell invasiveness (55). In addition, decreased ECE-1 expression, following ECE siRNA treatment, reduced ET-1–dependent oral squamous carcinoma cell proliferation (56). These reports support the approach proposed here, indicating that ECE-1 is a potential target for siRNA-mediated treatment of ovarian and other cancers either by i.p. injection or by novel delivery techniques that are being developed.

In summary, this study shows that ECE-1 siRNA is an effective tool for lowering ECE-1 levels and ET-1 biosynthesis in ovarian cancer. Consequently, ET-1 signaling and the invasive phenotype in ovarian cancer were reduced, thus suggesting its potential therapeutic use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

33. Klipper E, Gilboa T, Levy N, Kisloian T, Spanel-Bozorski K, Meidan R. Characterization of endothelin-1


Small Interfering RNA Molecules Targeting Endothelin-Converting Enzyme-1 Inhibit Endothelin-1 Synthesis and the Invasive Phenotype of Ovarian Carcinoma Cells

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