Endothelin-1 Is Required During Epithelial to Mesenchymal Transition in Ovarian Cancer Progression

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In a range of human cancers, tumorigenesis is promoted by activation of the endothelin A receptor (ET_AR)/endothelin-1 (ET-1) axis. ET-1 and ET_AR are overexpressed in primary and metastatic ovarian carcinomas, and high levels of ET-1 are detectable in patient ascites, suggesting that ET-1 may promote tumor dissemination. Moreover, in these tumors, engagement of ET_A receptor by ET-1 triggers tumor growth, survival, angiogenesis, and invasiveness. Thus, ET-1 enhances the secretion of matrix metalloproteinases, disrupts intercellular communications, and stimulates cell migration and invasion. Therefore, we investigated the role of the ET-1/ET_AR autocrine axis in promoting epithelial to mesenchymal transition (EMT) in ovarian tumor cells, a key event in cancer metastasis, in which epithelial cells depolarize, disassemble cell-cell contacts, and adopt an invasive phenotype. Here, we examine the potential role of ET-1 in regulating cell morphology and behavior and epithelial and mesenchymal proteins employing an *in vitro* 3-D culture system. We found that in 3-D serum-free collagen I gel cultures, HEY and OVCA 433 ovarian carcinoma cells undergo fibroblast-like morphologic changes between 3 and 5 days of ET-1 treatment. In these cells, ET-1 induces loss of adherens and tight-junction protein expression, E-cadherin, β-catenin, and zonula occludens-1, and gain of N-cadherin and vimentin expression. These results confirm the ability of ET-1 to promote EMT, a metastable process involving sustained loss of epithelial markers and gain of mesenchymal markers. Collectively, these findings provide evidence of a critical role for the ET-1/ET_AR axis during distinct steps of ovarian carcinoma progression, thus underlining this axis as a potential target in the treatment of ovarian cancer. Exp Biol Med 231:1128–1131, 2006

Key words: EMT; ovarian cancer; ET-1; ET_A receptor; invasion

Introduction

Epithelial ovarian cancer represents the most frequent cause of death among cancers of the female genital tract. Metastases to the peritoneum and stroma are common in this cancer and contribute to the overall poor outcome of ovarian cancer patients (1). Therefore, the development of new treatment protocols that control important processes in the tumor microenvironment could be a step forward in controlling these malignancies. The endothelin (ET) family is composed of three isopeptides, ET-1, -2, and -3, which act through two distinct subtypes of G-protein–coupled receptors, namely ET_A and ET_B. The ET_A receptor (ET_AR) is highly specific for ET-1, whereas ET_BR binds ET-1, ET-2, and ET-3 with the same affinity (2). ET-1 has been implicated in the pathophysiology of a variety of human tumors (3), including ovarian carcinoma (4, 5). ET-1 and the ET_AR are overexpressed in primary and metastatic ovarian carcinomas, and ET-1 is present at high levels in ovarian tumor ascites (5, 6). In ovarian tumor cells, ET-1 acts as an autocrine growth, survival, and angiogenic factor selectively through the ET_AR (4, 6–9) by activating diverse signaling pathways (10). These include mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase–dependent Akt activation, Src-mediated epidermal growth factor receptor transactivation (9), which is partly responsible for MAPK phosphorylation (11), and p125 focal adhesion kinase and paxillin activation, relevant to tumor cell invasion (10). ET-1, binding to ET_AR, consistently induces the activity of multiple metastasis-related proteinases, such as matrix-metalloproteinases (MMP) and the urokinase-type plasminogen activator system (12). Moreover, ET-1 inhibits gap junction intercellular communication by inducing phosphorylation of connexin 43, allowing tumor cells to escape growth control, and invade (13).

During recent years, epithelial to mesenchymal transition (EMT) has emerged as a central process during cancer progression and metastasization. During this process, cancer cells acquire a fibroblastoid invasive phenotype; down-regulate epithelial-specific proteins, such as adherens and

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tight junction proteins; induce various mesenchymal markers, such as vimentin, and finally migrate through the extracellular matrix (14, 15). Loss of E-cadherin protein and/or transcriptional repression of its mRNA are hallmarks of EMT (16, 17). Several studies indicated that down-regulation of E-cadherin is accompanied by upregulation of the mesenchymal N-cadherin expression, which correlates with invasion, metastasis, and EMT (18). The intracellular domain of E-cadherin interacts with catenin proteins, called α-, β-, γ-, and p120-catenin, which connect the adhesion complex to the actin cytoskeleton (19). The interaction between the cytoplasmic tail of cadherins with the catenins and the actin cytoskeleton is critical for the establishment of stable and functional adherens junction (AJ). Disruption of AJs releases β-catenin from the AJ pool. When it is not rapidly degraded, β-catenin translocates into the nucleus, where it induces the transcription not only of cyclin D1 and c-myc, but also of a variety of other genes that contribute to cancer progression, acting as a transcription factor in the nucleus by serving as a coactivator of the lymphoid enhancer factor (LEF)/TCF family of DNA-binding proteins. Thus, the loss of E-cadherin that causes disruption of cell adhesion and polarity allows tumor cell spreading, while the translocation of β-catenin into the nucleus might be required to induce the expression of genes that promote cell proliferation, invasion, and EMT (14). Additional events, including impaired proteasomal degradation of β-catenin, its tyrosine phosphorylation, and possibly a release from transcriptional inhibition, may be required to activate β-catenin signaling in cancer cells.

Many reports indicate that in addition to AJs, the modulation of the tight junctions (TJ) also has a significant impact on tumor development and metastasis and in the EMT. Zonula occludens-1 (ZO-1), a characteristic element of TJs, is a 220-kDa scaffolding protein containing various domains that allow its interactions with specialized sites of plasma membrane as well as with other proteins (20). Recently it has been demonstrated that in AJs, ZO-1 may functionally link E-cadherin with the cytoskeleton actin (21). Moreover, cytosolic localization of ZO-1 leads to phenotype alterations correlated to EMT as well as to an increased tumorigenicity in nude mice, most likely through a direct or indirect modulation of β-catenin/TCF/LEF signaling pathway (22).

In the context of tumorigenesis, EMT comprises a wide spectrum of changes in epithelial plasticity that can be induced by different agents and are dependent on particular cellular models. In ovarian carcinoma xenograft, ABT-627, a small molecule ET_AR antagonist with a suitable pharmacokinetic and toxicity profile for clinical use, achieves concomitant suppression of tumor growth and of various angiogenic and invasive determinants involved in tumor progression (23). In the present study, by using 3-D collagen gel cultures, we demonstrate for the first time that ET-1 is required to induce EMT in ovarian cancer cells, promoting a spindle-like and mesenchymal phenotype, involving loss of E-cadherin, β-catenin, and ZO-1 and acquisition of mesenchymal vimentin and N-cadherin. Thus, perturbing this complex signaling network during ovarian cancer progression by blocking the ET-1/ET_AR autocrine loop can be relevant as a new therapeutic approach in aggressive ovarian cancer.

Materials and Methods

Cell Culture. Human ovarian carcinoma cell lines, HEY and OVCA 433, were cultured as previously described (12). All culture reagents were from Invitrogen (Paisley, UK). Cells were cultured in serum-free medium for 24 hrs before ET-1 (100 n*M*; Peninsula Laboratories, Belmont, CA) stimulation.

3-D Collagen Gel Culture. Serum-free, 3-D cultures were performed as previously reported (15). Briefly, HEY cells (in serum-containing medium) and rat tail collagen (3–4 mg/ml, BD Transduction Laboratories, Heidelberg, Germany) were mixed rapidly at 0°C (final concentration 1.5 mg/ml), and 100-μl droplets containing 3500 cells dispensed into 17-mm wells. After solidification on a level surface at 23°C for 15–30 mins, the gels were incubated at 37°C in a CO_2 incubator for another 1–2 hrs and overlaid with 500 μl of serum-free medium containing ET-1 (100 n*M*), which was supplied with each medium change every day for a total of 5 days.

Immunoblotting. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and processed by immunoblotting using Ab to E-cadherin, N-cadherin, β-catenin (BD Transduction Laboratories), β-actin (Oncogene, Darmstadt, Germany), ZO-1 (Zymed Laboratories Inc., San Francisco, CA), and vimentin (Dako, Glostrup, Denmark). The proteins were visualized by ECL (Amersham, Arlington Heights, IL) and quantified using NIH Image (Scion, Frederick, MD).

Results

ET-1/ET_AR Autocrine Pathway Is Required for EMT. ET_AR overexpression is often accompanied in ovarian tumor cells by production of ET-1, and autocrine stimulation via ET-1 has been implicated in tumor progression (4, 5). Because of the unknown invasive responses, such as EMT, elicited by ET-1, we first analyzed cell plasticity of the two human ovarian cancer cell lines, HEY and OVCA 433, that release ET-1 and express functional ET_AR (4). The ET-1 levels released from HEY and OVCA 433 cells were within the physiologic range needed for activation of ET_AR in an autocrine fashion (Fig. 1A). To analyze cell morphology and behavior and epithelial and mesenchymal markers after ET-1 treatment, we used a 3-D collagen I culture system. These culture conditions have allowed the analysis of ovarian cancer cells responses to ET-1 in a more physiological way than is possible in conventional 2-D cultures. In contrast to scattering, full EMT is completed only after more than 3–6 days of exposure to

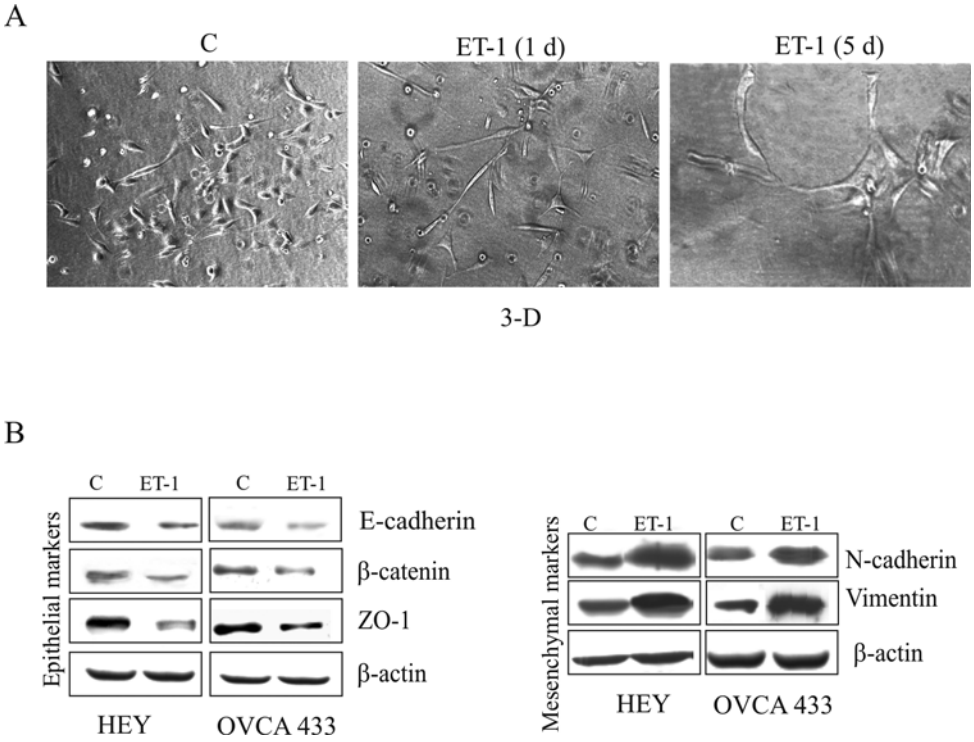

Figure 1. (A) Ovarian cancer cells are induced by ET-1 to undergo EMT. HEY cells were grown in the absence or presence of ET-1 (100 n*M*) and were seeded into collagen gels for 1 or 5 days. Morphologic changes were observed by phase-contrast microscope and photographs of representative cultured cells are shown. (B) ET-1 promotes the loss of epithelial markers and gain of mesenchymal markers. Serum-starved HEY and OVCA 433 cells were untreated or treated with 100 n*M* ET-1. Expression of epithelial proteins, including E-cadherin, β-catenin, ZO-1, and mesenchymal proteins including vimentin and N-cadherin was examined by immunoblotting. β-actin was used as a loading control.

several signals, and occurs only in certain cell types, some of which require 3-D culture conditions to rapidly and synchronously undergo EMT. As shown in Figure 1A, ET-1 (100 n*M*) induced morphologic changes including dissociation from neighboring cells. After 1 or 5 days of ET-1 treatment, HEY cells underwent morphologic changes compatible with EMT: they became elongated and spindle-shaped, with a fibroblastoid morphology.

To determine whether the molecular alterations of an EMT occurred in these cells, we examined the expression of epithelial and mesenchymal markers by immunoblotting. In lysates from serum-starved HEY and OVCA 433 cultured in 3-D collagen I with ET-1 (100 n*M*) for 5 days, we observed a loss of membrane expression of E-cadherin, β-catenin, and the tight-junction molecule ZO-1, indicating a reduction in epithelial marker expression. In contrast, the expression of fibroblast markers, including vimentin and membrane N-cadherin, was strongly induced (Fig. 1B). Both the morphologic and the molecular changes demonstrated that ovarian cancer cells, after prolonged ET-1 exposure, had undergone EMT.

Discussion

Metastasis is a complex, multistep process, involving basement membrane destruction and local invasion, intravasation and survival in the bloodstream, extravasation into distant organs, and survival plus proliferation at the metastatic site. The very recent recognition of EMT as a potential mechanism for metastasis, and the increasingly detailed knowledge of the underlying molecular processes that trigger this process, may offer new targets for therapeutic intervention (15).

We recently demonstrated that in melanoma cells, activation of ET_BR by ET-1 induces downregulation of E-cadherin and associated catenin proteins, such as β-catenin and p120-catenin, with a parallel upregulation of N-cadherin. This latter change allows homotypic adhesive contact as well as heterotypic (i.e., fibroblasts and endothelial cells) melanoma cell-cell interactions. We also identified one pathway regulating transcription of E-cadherin. In melanoma cells, ET-1 induces a significant upregulation of *Snail* mRNA and a concomitant E-cadherin mRNA downregulation. In addition, ET-1 can suppress the adherent junctional function through the tyrosine phosphorylation of β- and p120-catenins, resulting in increased levels of catenin-free pools that cause a decreased cell-cell adhesion. Importantly, pharmacologic blockade of ET_BR inhibits tumor xenograft growth and EMT molecular determinants (24).

In the present study, using a 3-D collagen culture as a new experimental approach to identify epithelial-plasticity changes in tumor cells closely resembling *in vivo* effects, we demonstrated for the first time that in ovarian cancer cells, ET-1 is essential for a complete EMT phenotype. In this

condition, chronic ET-1 exposure induces ovarian cancer cells to lose their polarized phenotype, becoming migratory and fibroblastoid in shape. Molecular analyses of these cells demonstrated that ET-1 destabilized tight and adherens junctions by downregulating the expression of ZO-1, E-cadherin, and β-catenin with concomitant induction of mesenchymal markers such as vimentin and N-cadherin. These results were consistent with the previous observation, and complement and extend the identification of ET-1 as a key growth factor that activates cell signaling controlling EMT and tumor progression. In this context, ET-1/ET_AR induces the disruption of normal host-tumor interactions regulating changes in cadherin, connexin, and MMP expression (13, 23). Indeed, the immunohistochemical and immunoblot analysis of HEY xenografts provides *in vivo* evidence for this concept, demonstrating that treatment with ABT-627 induces a significant reduction in microvessel density, a reduced expression of vascular endothelial growth factor, cyclooxygenase-2, and MMP-2, and increased tumor apoptosis and gap junctional intercellular plaques (9, 13, 23). The ET-1/ET_AR autocrine loop is present in a significant percentage of aggressive ovarian tumors, indicating that this loop is highly relevant in promotion of EMT, invasion, and metastasis in ovarian cancer. Thus, the ability of ET_AR antagonists to induce concomitant suppression of tumor cell proliferation and/or survival (7, 23), and simultaneously control EMT, provides a rationale for developing more effective therapeutic intervention in aggressive ovarian cancer.

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