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Heterogeneous Cadherin Expression and Multicellular Aggregate Dynamics in Ovarian Cancer Dissemination

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Abstract

Epithelial ovarian carcinoma spreads via shedding of cells and multicellular aggregates (MCAs) from the primary tumor into peritoneal cavity, with subsequent intraperitoneal tumor cell:mesothelial cell adhesion as a key early event in metastatic seeding. Evaluation of human tumor extracts and tissues confirms that well-differentiated ovarian tumors express abundant E-cadherin (Ecad), whereas advanced lesions exhibit upregulated N-cadherin (Ncad). Two expression patterns are observed: "mixed cadherin," in which distinct cells within the same tumor express either E- or Ncad, and "hybrid cadherin," wherein single tumor cell(s) simultaneously expresses both cadherins. We demonstrate striking cadherin-dependent differences in cell-cell interactions, MCA formation, and aggregate ultrastructure. Mesenchymal-type Ncad+ cells formed stable, highly cohesive solid spheroids, whereas Ecad+ epithelial-type cells generated loosely adhesive cell clusters covered by uniform microvilli. Generation of "mixed cadherin" MCAs using fluorescently tagged cell populations revealed preferential sorting into cadherin-dependent clusters, whereas mixing of cell lines with common cadherin profiles generated homogeneous aggregates. Recapitulation of the "hybrid cadherin" Ecad+/Ncad+ phenotype, via insertion of the *CDH2* gene into Ecad+ cells, resulted in the ability to form heterogeneous clusters with Ncad+ cells, significantly enhanced adhesion to organotypic mesomimetic cultures and peritoneal explants, and increased both migration and matrix invasion. Alternatively, insertion of *CDH1* gene into Ncad+ cells greatly reduced cell-to-collagen, cell-to-mesothelium, and cell-to-peritoneum adhesion. Acquisition of the hybrid cadherin phenotype resulted in altered MCA surface morphology with increased surface projections and increased cell proliferation. Overall, these findings support the hypothesis that MCA cadherin composition impacts intraperitoneal cell and MCA dynamics and thereby affects ultimate metastatic success.

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Abbreviations: 3D, three-dimensional; BSA, bovine serum albumin; CMFDA, green 5-chloromethylfluorescein diacetate; CMTPX, 4-({[4-(chloromethyl)phenyl]carbonyl}amino)-2-(1,2,2,4,8,10,10,11-octamethyl-10,11-dihydro-2H-pyrano[3,2-g:5,6-g′]diquinolin-1-ium-6-yl)benzoate; Ecad, E-cadherin; ECM, extra-cellular matrix; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; EMT, epithelial-to-mesenchymal transition; EOC, epithelial ovarian carcinoma; FBS, fetal bovine serum; hGAPDH, housekeeping glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; MCA, multicellular aggregate; MEM, minimal essential medium; MET, mesenchymal-to-epithelial transition; Ncad, N-cadherin; NEAA, non-essential amino acids; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; RFP, red fluorescent protein; RTCI, rat tail collagen type I; SEM, scanning electron microscopy; SFM, serum-free medium; TEM, transmission electron microscopy; TMA, tissue microarray.

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Introduction

Epithelial ovarian carcinoma (EOC) is the fifth leading cause of cancer-related death among women and the most lethal of all gynecological malignancies with 22,280 new cases and 14,240 deaths estimated in the United States in 2016 [1]. The location and size of the ovaries and fallopian tubes, relatively nonspecific symptoms, and absence of reliable screening methods make EOC difficult to detect in early stages and worsen the survival prognosis. The vast majority of women are diagnosed with stage III to IV disease with involvement of distant organs and lymph nodes, resulting in a 5-year relative survival rate of about 25%. In contrast, women with localized cancer whose lesions are limited to primary tumor site have a 95% 5-year survival [2]. Notably, most patients initially respond very well to chemotherapy but later relapse with more advanced, multidrug-resistant metastatic disease [3,4]. Therefore, it is crucial to gain understanding of the mechanisms involved in metastatic success in order to develop novel therapeutic approaches that could improve long-term survival.

In contrast to most other tumors that spread predominantly through lymph or bloodstream, ovarian cancer disseminates mainly via direct extension of cancer cells from the primary tumor into the intra-abdominal cavity, wherein they survive and travel as single cells and multicellular aggregates (MCAs) with the peritoneal fluid flow, subsequently adhering to peritoneal tissues, anchoring in the submesothelial matrix, and proliferating to form secondary lesions [5,6]. Hematogenous metastasis has also been described [7,8]. Free-floating EOC aggregates are abundant in the peritoneal cavity; however, the number, size, and integrity of aggregates vary considerably [9,10]. Although free-floating single cells and MCAs were previously considered as nonadhesive units that do not contribute to metastatic progression, it is now generally accepted that MCAs are indeed metastatically competent and can actively adhere to peritoneal mesothelium and submesothelial extracellular matrix [11]. Nevertheless, most patient-derived MCAs that adhere to and disperse on extracellular matrix components (such as collagen type I) and mesothelial cell layers display limited invasion, suggesting the existence of a subpopulation of MCAs with enhanced metastatic properties [12]. Due to difficulties associated with accessing human peritoneal effusion samples together with the technical challenges involved in maintenance and manipulation of primary patient-derived MCAs, these clinically relevant metastatic units remain understudied. Currently, techniques are available for generation of three-dimensional (3D) clusters from immortalized cell lines *in vitro,* which have been used predominantly to address hypoxia-, anoikis-, and chemoresistance [13–19]. The basic biology of MCA dynamics and cellular characteristics that dictate a metastatically successful MCA phenotype has yet to be elucidated.

Cadherins are a superfamily of calcium-dependent transmembrane adhesion molecules which mediate cell-cell adhesion, and maintain monolayer integrity and normal tissue architecture throughout the organism. In most epithelial tissues E-cadherin (Ecad) is responsible for the maintenance of cell-cell junctions. Loss of Ecad expression together with the acquisition of N-cadherin (Ncad) expression, designated epithelial-to-mesenchymal transition, is often associated with tumor progression and stromal invasion [20–22]. Mesenchymal-to-epithelial transition may be observed after extravasation and metastatic colonization [22]. In the healthy ovary, however, the mesodermally derived normal ovarian surface epithelium junctions are maintained exclusively by Ncad, whereas Ecad conditional coexpression is thought to be a sign of metaplasia [5,20,23–25]. Well-differentiated ovarian tumors express abundant Ecad, whereas advanced-stage and metastatic lesions exhibit upregulated Ncad expression [5,24–26]. Recent data also suggest human fallopian tube secretory epithelium as a possible progenitor of high-grade serous ovarian malignancies [27]. Normal tubal epithelial cells exhibit continuous expression of Ecad together with Ncad, whereas Ncad is lost in impaired atrophic tubal regions [28,29]. It has been reported that acquisition of Ecad enables MCAs to avoid detachment-induced apoptosis and resist radiation and chemotherapy [30–33]. Conversely, loss of Ecad via transcriptional or proteolytic mechanisms, induced by lysophosphatidic acid– or epidermal growth factor–related signaling, leads to increased detachment of EOC cells from the tumor surface [34–37]. As the contribution of cadherin switching to regulation of MCA dynamics and peritoneal invasion has not been examined, the objective of the current study is to comprehensively characterize the contribution of cadherin profiles to the behavior of free-floating EOC single cells and MCAs.

Materials and Methods

Cell Lines

The epithelial ovarian carcinoma cell lines OvCa433, OvCa429, OvCa432, and DOV13 were provided by Dr. Robert Bast (M.D. Anderson Cancer Center, Houston, TX) and maintained in minimal essential medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 1% nonessential amino acids (Corning Cellgro), 1% penicillin/streptomycin (Lonza), 1% sodium pyruvate (Corning Cellgro), and 0.1% amphotericin B (Cellgro); DOV13 medium was additionally supplemented with 10 μg/ml of insulin (Gibco). The ovarian adenocarcinoma OVCAR3 and SKOV3 cell lines were purchased from American Type Culture Collection (Manassas, VA). OVCAR3 cell line was maintained in RPMI 1640 medium (Gibco) supplemented with 20% FBS, 1% sodium pyruvate, 1% nonessential amino acids, 1% GlutaMAX (Gibco), 1% penicillin/streptomycin, and 0.1% amphotericin B. SKOV3 cells were maintained in McCoy's 5a medium (Gibco) containing 10% FBS, 1% penicillin/streptomycin, 1% GlutaMAX, and 0.1% amphotericin B. Human mesothelial LP9 cell line was obtained from Coriell NIA Aging Cell Repository (Camden, NJ) and maintained in a 1:1 ratio of M199 and Ham F12 media (Gibco), supplemented with 15% FBS, 1% penicillin/streptomycin, 1% HEPES (Gibco), 1% GlutaMAX, 10 ng/ml of epidermal growth factor (Sigma), and 400 ng/ml of hydrocortisone. Cell lines were tested and authenticated by Genetica DNA Laboratories using short tandem repeat DNA profiling and were found to be >95% concordant. Red fluorescent protein (RFP) lentiviral vector (GenTarget, San Diego, CA) and green fluorescent protein (GFP) lentiviral vector (AddGene, Cambridge, MA) were utilized to create tagged OvCa433-RFP and SKOV3-GFP stable cell lines, respectively. Lentiviral transductions were performed according to manufacturers' protocols, and successfully labeled cells were further selected via BD FACSAria III cell sorter.

Construction of a pmCherry:Ncad Plasmid

pmCherry-N1 vector was obtained from Clontech Laboratories, Inc. (Mountain View, CA). The murine Ncad–enhanced GFP construct was a gift from Dr. Cecile Gauthier-Rouviea. Ncad cDNA was subcloned into the pmCherry-N1 ApaI/AgeI sites using enzymes purchased from NEB, Inc. (Ipswich, MA). Gel purification of both the vector and insert was accomplished using the QiaQuick Gel