Ectodomain shedding of the cell adhesion molecule Nectin-4 in ovarian cancer is mediated by ADAM10 and ADAM17

Petra C. Buchanan†, Kristin L. M. Boylan‡, Bruce Walcheck shoppers, Rachel Heinze¶, Melissa A. Geller§, Peter A. Argenta☆, and Amy P. N. Skubitz†

From the Departments of †Laboratory Medicine and Pathology, ¶Veterinary and Biomedical Sciences, and §Obstetrics, Gynecology, and Women’s Health, University of Minnesota, Minneapolis, Minnesota 55455

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We previously showed that the cell adhesion molecule Nectin-4 is overexpressed in ovarian cancer tumors, and its cleaved extracellular domain can be detected in the serum of ovarian cancer patients. The ADAM (a disintegrin and metalloproteinase) proteases are involved in ectodomain cleavage of transmembrane proteins, and ADAM17 is known to cleave Nectin-4 in breast cancer. However, the mechanism of Nectin-4 cleavage in ovarian cancer has not yet been determined. Analysis of ovarian cancer gene microarray data showed that higher expression of Nectin-4, ADAM10, and ADAM17 is associated with significantly decreased progression-free survival. We quantified Nectin-4 shedding from the surface of ovarian cancer cells after stimulation with lysophosphatidic acid. We report that ADAM17 and ADAM10 cleave Nectin-4 and release soluble Nectin-4 (sN4). Small molecule inhibitors and siRNA knockdown of both ADAM proteases confirmed these results. In matched samples from 11 high-grade serous ovarian cancer patients, we detected 2–20-fold more sN4 in ascites fluid than serum. Co-incubation of ovarian cancer cells with ascites fluid significantly increased sN4 shedding, which could be blocked using a dual inhibitor of ADAM10 and ADAM17. Furthermore, we detected RNA for Nectin-4, ADAM10, and ADAM17 in primary ovarian carcinoma tumors, secondary omental metastases, and ascites fluid from serous ovarian cancer patients. In a signaling pathway screen, lysophosphatidic acid increased phosphorylation of AKT, EGF receptor, ERK1/2, JNK1/2/3, and c-Jun. Understanding the function of Nectin-4 shedding in ovarian cancer progression is critical to facilitate its development as both a serum biomarker and a therapeutic target for ovarian cancer.

Ovarian cancer is the most lethal gynecologic malignancy, with 21,000 estimated new cases and over 14,000 associated deaths annually in the United States (1). Currently, early detection tests are lacking, and as a result most women are diagnosed at late stages, portending a poor prognosis.

Ovarian cancer spreads primarily through localized detachment from the primary tumor, which may arise in the fimbriae of the fallopian tube (2) and seed within the peritoneal cavity; however, hematogenous metastasis also occurs (3). Ovarian cancer can exist both as free-floating single cells in the peritoneal cavity (ascites fluid) or as spheroid multicellular aggregates. Spheroids first adhere to extracellular matrix proteins and monolayers of mesothelial cells (4, 5), followed by disaggregation and invasion of the peritoneal organs (6).

Normal tissue expression of the cell adhesion molecule Nectin-4 (PVRL4, or poliovirus receptor-related 4) is largely limited to the placenta, with lower levels of expression in the skin, stomach, esophagus, breast, bladder, prostate, lung, and trachea (7). We discovered the overexpression of Nectin-4 in ovarian cancer tissues by gene microarray analysis (8), and we subsequently showed both Nectin-4 RNA and protein overexpression in ovarian cancer tissues compared with their normal ovarian counterparts and in various cell lines (9). A recent study found increased Nectin-4 mRNA expression in over 97% of ovarian cancer samples (10). Furthermore, overexpression of Nectin-4 has been described in ductal breast carcinoma, lung adenocarcinoma, and pancreatic cancer and is associated with disease progression or poor prognosis (11–13).

The Nectin family is important in the formation and maintenance of adherens and tight junctions (14), but the role of Nectin-4 in cellular functions beyond cell-cell adhesion is not well understood. We recently reported that Nectin-4 promotes cell-cell adhesion and the formation of spheroids and leads to increased cell migration, as well as proliferation in ovarian cancer cell lines (15). Nectin-4 promotes anchorage independence in breast cancer cells (16), and the extracellular domain of some Nectins can bind to growth factor receptors, which may play a role in the regulation of cell proliferation, migration, and apoptosis (17).

The extracellular domain of Nectin-4 can be proteolytically cleaved to release a soluble fragment (sN4), which may regul-

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† To whom correspondence should be addressed: Dept. of Laboratory Medicine and Pathology, University of Minnesota, MMC 395, 420 Delaware St., S.E., Minneapolis, MN 55455. Tel.: 612-625-5920; Fax: 612-625-0665; E-mail: skubi002@umn.edu.

2 The abbreviations used are: sN4, soluble Nectin-4; N4, Nectin-4; ADAM, a Disintegrin and Metalloproteinase; EGFR, EGF receptor; EGA, European Genome-phenome Archive; TCGA, The Cancer Genome Atlas; PMA, phorbol 12-myristate 13-acetate; LPA, lysophosphatidic acid; PFS, progression-free survival; MMP, matrix metalloproteinase.
Ectodomain shedding of Nectin-4 in ovarian cancer

We have previously shown that the human ovarian cancer cell line NIH:OVCAR5 expressing moderate levels of Nectin-4 (9, 15). To facilitate detection and quantification of the extracellular sN4, NIH:OVCAR5 parental cells were transfected with full-length Nectin-4, and cells that overexpressed Nectin-4 (NIH:OVCAR5-N4-over) were selected using FACS. Approximately 95% of the NIH:OVCAR5 parental cells (Fig. 2A) expressed Nectin-4, and 99% of the NIH:OVCAR5-N4-over cells expressed Nectin-4 (Fig. 2B). A distinct population comprising ~75% of the NIH:OVCAR5-N4-over cells expressed 100 times more Nectin-4 than the parental cells. Over 99% of the parental and NIH:OVCAR5-N4-over cells expressed both ADAM17 and ADAM10 (Fig. 2).

**Ectodomain shedding of Nectin-4 can be stimulated in a time-dependent manner**

We stimulated Nectin-4 ectodomain shedding from the surface of NIH:OVCAR5-N4-over cells with both phorbol 12-myristate 13-acetate (PMA) and lysophosphatidic acid (LPA). Although PMA works as a pleiotropic stimulator and does not have physiological relevance, many researchers have used PMA to analyze shedding of different substrates (30). LPA was used as a physiologic stimulus, because it is known to be present in high amounts in the ascites fluid of ovarian cancer patients. Shed Nectin-4 was then quantified in the culture supernatant by ELISA.

**NIH:OVCAR5 cells express ADAM17 and ADAM10 on their surface**

| Nectin-4 | ADAM17 | ADAM10 |
|----------|--------|--------|
| ![Graph A](image)
| ![Graph B](image)
| ![Graph C](image)

Figure 1. Progression-free survival analysis of serous ovarian cancer patients. The Kaplan-Meier plotter for ovarian cancer was used to plot progression-free survival data of grade 1 and 2 serous ovarian cancer patients relative to gene expression. The program combines Affymetrix microarray data from the EGA and TCGA. The PFS data were shown for Nectin-4 (p = 0.048, A), ADAM17 (p = 0.039, B), and ADAM10 (p = 0.014, C). Lower expression is shown as a black line, and higher expression is shown as a red line. The respective Affymetrix IDs used for the calculations were 223540_at (Nectin-4), 213532_at (ADAM17), and 202604_x_at (ADAM10).

Results

Higher expression of Nectin-4, ADAM10, and ADAM17 is associated with decreased progression-free survival

We analyzed progression-free survival (PFS) data of grade 1 and 2 serous ovarian cancer patients using the Kaplan-Meier plotter for ovarian cancer, which incorporates data from Affymetrix microarrays from the European Genome-phenome Archive (EGA), as well as The Cancer Genome Atlas (TCGA) (29). PFS data were plotted for Nectin-4, ADAM17, and ADAM10, respectively (Fig. 1, A–C). Higher expression of each of these three genes was associated with significantly decreased PFS (p < 0.05 in all cases).

Late cellular functions (18). Two studies detected sN4 in the serum of breast and lung cancer patients, and a correlation between high levels of sN4 and the number of metastases was shown in breast cancer (13, 19). Our group and others showed that serum levels of Nectin-4 correlate with CA-125 levels in ovarian cancer patients, whereas in patients with benign gynecologic diseases with elevated serum levels of CA-125, Nectin-4 was very low or not detected (9, 10).

In humans, the ADAM (a disintegrin and metalloproteinase) family of proteases comprises 22 proteins, of which only half have proteolytic activity (20). The expression of ADAM proteins varies; many are exclusively or predominantly expressed in the testis or associated structures, others are broadly somatically expressed (21). The role of the ADAM proteases in cellular function is multifaceted. Ectodomain shedding of membrane proteins by ADAMs regulates cell proliferation, adhesion, and migration and is involved in cell signaling. They play important roles in cancer formation, progression, and resistance to therapy (20). Substrates for ADAMs include growth factors, cytokines, receptors, as well as adhesion molecules, including EGFR ligands, TNF-α, CD16, and CD44 (22–25).

A recent study identified ADAM10 to be involved in the progression of breast cancer, where high expression was associated with poor prognosis (26). ADAM10 overexpression was also detected in liver cancer and was shown to stimulate proliferation, migration, and invasion of hepatocellular carcinoma cells (27). In breast cancer, ADAM17 is involved in cancer progression and predicts adverse outcome (28). In this study, we investigated the role of ADAM10 and ADAM17 in ectodomain shedding of the cell adhesion molecule Nectin-4 in the NIH:OVCAR5 ovarian cancer cell line, patients’ serum, ascites fluid, and solid tumor samples.
ding of Nectin-4 was also observed after 3 h of culture and increased 2-fold after 6 h of culture (Fig. 3B).

**Specific inhibitors of ADAM17 and ADAM10 block ectodomain shedding of Nectin-4**

To determine whether ADAM17 and ADAM10 contribute to the ectodomain shedding of Nectin-4, various inhibitors (Table 1) were used before stimulating the NIH:OVCAR5-N4-over cells with either PMA (Fig. 3C) or LPA (Fig. 3D). The broad-spectrum matrix metalloprotease (MMP) inhibitor BB-94 (Batimastat) (31) was used as a positive control and strongly blocked shedding of Nectin-4 after stimulation with PMA (96%) or LPA (82%). INCB3619 is a selective dual inhibitor for both ADAM17 and ADAM10 (32) and showed strong activity in blocking Nectin-4 shedding, after both PMA stimulation (94%) and LPA stimulation (81%). BMS566395, referred to as inhibitor 32 in Ref. 33, is a selective inhibitor of ADAM17 (34) and inhibited shedding by 70 and 27% following stimulation with PMA or LPA, respectively. The functional ADAM17 blocking antibody D1(A12) (35) showed similar inhibition patterns as BMS566395 but was slightly less potent at inhibiting the shedding of Nectin-4 after stimulation with PMA (47%). Although shedding was reduced with LPA stimulation (12% inhibition), it did not reach statistical significance. GI254023X is reported to be 10-fold more selective for inhibiting ADAM10 than ADAM17 in published cellular assays (30, 34, 36) and showed limited, albeit statistically significant, inhibition after stimulation with PMA (14%) while blocking Nectin-4 shedding by 55% after physiologic stimulation with LPA.

Of note, the inhibitors BB-94, INCB3619, and GI254023X were able to partially block constitutive shedding of Nectin-4 by ~70, 80, and 20–40%, respectively. However, the inhibitor BMS566395 and the human antibody D1(A12), both inhibitors of ADAM17, did not inhibit constitutive shedding.

**siRNA-mediated knockdown of ADAM proteases inhibits shedding of Nectin-4**

To confirm our results using the specific inhibitors of ADAM17/10, we analyzed shedding of Nectin-4 by NIH:OVCAR5-N4-over cells after siRNA-mediated knockdown of both ADAM proteases. Densitometry of the duplex RT-PCR for GAPDH and ADAM17 (Fig. 4A) or GAPDH and ADAM10 (Fig. 4B) showed ~90% knockdown for each protease 48 h after transfection. The siRNA for ADAM17 only slightly reduced mRNA amounts of ADAM10 compared with the negative control (15%). A negative control pool of siRNAs and a GAPDH-positive control showed no knockdown of either ADAM17 (Fig. 4A) or ADAM10 (Fig. 4B). When both proteases were targeted simultaneously, 90% knockdown of each ADAM was observed.

After 48 h in culture with siRNAs, ADAM10 and/or ADAM17 knockdown cells were stimulated with PMA for 3 h, and shedding of Nectin-4 was quantified relative to the shedding observed with the negative control siRNA pool (Fig. 4C). Knockdown of ADAM17 alone reduced shedding by 61%; adding the ADAM10 inhibitor GI254023X reduced shedding by a total of 77%. Knockdown of ADAM10 alone reduced shedding by 28%; adding the ADAM17 inhibitor BMS566395 blocked Nectin-4 shedding by a total of 89%. A combined...
knockdown of ADAM10 and ADAM17 led to a reduction in Nectin-4 shedding by 77%; adding the double inhibitor INCB3619 inhibited shedding by a total of 97%. Physiologic stimulation of cells with LPA (Fig. 4) after ADAM17 knockdown reduced Nectin-4 shedding by 32%; adding the ADAM10 inhibitor GI254023X reduced shedding by a total of 55%. Knockdown of ADAM10 reduced shedding by almost 40%; adding BMS566395 to inhibit ADAM17 reduced shedding by a total of 68%. The double knockdown of ADAM17/10 blocked shedding by 56%; adding the dual inhibitor INCB3619 blocked shedding of Nectin-4 by a total of 93%.

Levels of shed Nectin-4 are higher in patients’ ascites than in serum samples

We quantified the amounts of sN4 in matched ascites and serum samples from 11 high-grade serous ovarian cancer patients. Levels of sN4 varied greatly (Fig. 5A) but in general were higher in the ascites fluid than in the respective serum sample. For example, patient 1 had the highest levels of sN4 (>13 ng/ml in ascites and >1.6 ng/ml in serum), patient 6 had medium levels of sN4 (>5 ng/ml in ascites and >1.6 ng/ml in serum), whereas patients 10 and 11 had the lowest levels of sN4 (<1 ng/ml but still detectable in both ascites and sera).

Co-incubation of NIH:OVCA5 cells with patient ascites stimulates shedding of Nectin-4

Four of the above characterized ascites samples were co-incubated with NIH:OVCA5-N4-over cells for 3 h, and shedding of Nectin-4 was quantified by ELISA. All four ascites samples significantly increased sN4 shedding (Fig. 5B, white triangles) over the baseline level of sN4 detected in ascites fluid (incubated in wells without cells, Fig. 5B, black circles). Ascites
from patient 1, which had the highest amount of Nectin-4, increased sN4 levels 2.5-fold. Ascites from patient 11, which had the lowest amount of Nectin-4, stimulated sN4 shedding by 38-fold. Patients 4 and 6 had medium amounts of sN4 in their ascites samples and increased shedding 4- and 5-fold, respectively. When the dual ADAM10 and ADAM17 inhibitor INCB3619 was added to the cells before stimulation with the ascites fluid, the levels of sN4 decreased almost to baseline levels (Fig. 5B, white squares).

Expression of Nectin-4, ADAM10, and ADAM17 in ovarian cancer patient tissues

We analyzed expression levels of Nectin-4, ADAM10, and ADAM17 by RT-PCR in matched samples from four ovarian cancer patients (Fig. 5C). Two samples (patient 6 and 11) had been analyzed for their sN4 levels in Fig. 5A and were used for stimulating NIH:OVCAR5-N4-over cells in Fig. 5B. The primary tumor and the ascites cells of all four patients expressed Nectin-4 and both ADAM proteases in varying amounts, compared with the β-actin loading control. Patient 6 had more Nectin-4 mRNA than patient 11 in tissues tested, which could explain the higher levels of sN4 observed in ascites fluid and serum compared with patient 11 (Fig. 5A). The omental tissue samples contained less mRNA for Nectin-4, ADAM10, and ADAM17 than the primary tumor and ascites cells in half of the cases (patients 11 and 12).

Nectin-4 expression affects LPA-stimulated signaling pathways

To determine the role of Nectin-4 shedding in cell signaling, we interrogated two antibody arrays specific for the phosphorylated forms of 43 human phosphokinase and 49 receptor tyrosine kinase molecules. NIH:OVCAR5 cells with shRNA-mediated knockdown of Nectin-4 expression (NIH:OVCAR5-N4-KD) and control NIH:OVCAR5 cells were serum-starved overnight and then stimulated with LPA for 1 h and compared with unstimulated cells. In response to stimulation with LPA, the phosphorylation of five signaling molecules (AKT, EGFR, ERK1/2, JNK1/2/3, and c-Jun) was substantially increased in the NIH:OVCAR5 control cells (Fig. 6, A–F). Phosphorylation of the human phosphokinase AKT was examined at two sites, Ser-473 and Thr-308. Phosphorylation of AKT at Ser-473 was strongly increased in the response to LPA stimulation of the NIH:OVCAR5 control cells (Fig. 6A). However, there was no difference in LPA-stimulated phosphorylation in the NIH:OVCAR5-N4-KD cells (Fig. 6A), suggesting that Nectin-4 expression is required for the LPA-stimulated increase in AKT phosphorylation at this activating site. In contrast, low levels of...
phosphorylation were detected at Thr-308, and there was no difference in phosphorylation between the NIH:OVCAR5 control or NIH:OVCAR5-N4-KD cells, with or without LPA stimulation (Fig. 6B).

We also observed that phosphorylation of the growth factor receptor EGFR was over 6-fold lower in the NIH:OVCAR5-N4-KD cells compared with the control cells. Treatment with LPA increased EGFR phosphorylation in both the control and NIH:OVCAR5-N4-KD cells; however, even with LPA stimulation, EGFR phosphorylation was 3-fold lower in the NIH:OVCAR5-N4-KD cells compared with the control cells (Fig. 6C). A similar pattern of phosphorylation was observed for ERK1/2, where phosphorylation was low in the NIH:OVCAR5-N4-KD cells but was sensitive to LPA stimulation in the presence and absence of Nectin-4 expression (Fig. 6D). In contrast, constitutively low levels of JNK1/2/3 and c-Jun phosphorylation were observed in both the control and NIH:OVCAR5-N4-KD cells (Fig. 6, E and F, respectively). Phosphorylation of both molecules was stimulated by LPA ~3-fold in the control NIH:OVCAR5 cells that express Nectin-4, whereas stimulation by LPA in the NIH:OVCAR5-N4-KD cells resulted in less than a 2-fold increase in phosphorylation (Fig. 6, E and F).

Differences in the phosphorylation of WNK1, Chk-2, and β-catenin were also observed between control and NIH:OVCAR5-N4-KD cells; however, the phosphorylation of these molecules was not affected by LPA stimulation (data not shown).
shown). In contrast, CREB phosphorylation was stimulated by LPA but was not affected by Nectin-4 expression (data not shown). The constitutive levels of phosphorylation observed with the remaining 83 molecules tested in the screening assay were very low; thus, it was not possible to make definitive conclusions as to whether LPA stimulation or Nectin-4 expression affected their phosphorylation.

Discussion

Our findings are the first to demonstrate that both ADAM10 and ADAM17 are major sheddases of Nectin-4 from the surface of ovarian cancer cells. We looked at the influence of Nectin-4, ADAM10, and ADAM17 on the PFS of grade 1 and 2 serous ovarian cancer patients. Higher expression of each of these genes was associated with significantly decreased PFS. We also analyzed the TCGA data in the cBio Portal (37, 38), and although alterations of these three genes were uncommon (3–5%), two particular case sets showed significantly decreased disease-free survival rates: the mesenchymal cluster and the methylation cluster 3 (Ref. 39 and data not shown). Further studies are needed to determine whether these patients would benefit from therapy that targets Nectin-4 and ADAM10/17.

ADAM proteases can be activated by diverse mechanisms, for example by reduction-oxidation modifications (40, 41), activation of PKC through PMA (42), calcium ionophores (22), and G-protein-coupled receptors such as LPA receptors (43). Stimulation with PMA does not reflect the physiologic activity (44), but because many articles on ADAM proteases use PMA to stimulate cells and to analyze shedding of different substrates (30), this is an established approach to which we could compare our results. Our study also looked at physiologic stimulation with LPA, which is secreted by ovarian cancer cells and peritoneal mesothelial cells in vivo, leading to increased adhesion, migration, and invasion of ovarian cancer cells (45). High levels of LPA have been detected in the ascites fluid of ovarian cancer patients compared with patients with benign tumors and healthy controls, and LPA levels correlate with tumor aggressiveness (46).

ADAM10 and ADAM17 are similar in terms of amino acid sequence and structure (47, 48), yet they are differentially regulated (23). Several substrates have been shown to be cleaved by both ADAMs (23, 30, 36). In addition, either enzyme can potentially compensate for the other in terms of ectodomain shedding (44); therefore it is important to examine the role of both proteases.
Ectodomain shedding of Nectin-4 in ovarian cancer

Our study shows that ADAM10 and ADAM17 are major proteases for Nectin-4 shedding after stimulation with LPA. Combined ADAM10/ADAM17 inhibition or broad-spectrum inhibition before LPA stimulation reduced Nectin-4 shedding by over 80%. ADAM10 inhibition alone reduced shedding by 55%, whereas ADAM17 inhibition reduced Nectin-4 shedding by only 12–27%. In our studies, ADAM10 played a more dominant role than ADAM17 in Nectin-4 shedding after physiologic stimulation with LPA, in contrast to breast cancer, where ADAM17 was shown to be the major sheddase involved in Nectin-4 shedding; however, in those studies cells were only stimulated with PMA (18). Of note, ADAM17 is known to primarily respond to PMA (49), which was confirmed in our study. We observed that constitutive Nectin-4 shedding could be inhibited by all ADAM10 inhibitors, whereas the ADAM17 inhibitors had little effect. This suggests that ADAM10 is involved in the constitutive shedding of Nectin-4, which confirms reports of ADAM10 mediating low-level constitutive shedding of other cell surface molecules, whereas ADAM17 mediates high-level inducible shedding (30).

Although trans cleavage by ADAMs may be possible (50, 51), it is not the primary mechanism for most substrates. Rather, for most ADAM proteases, cleavage in cis is the predominant shedding mechanism (52, 53). In our study, we show by flow cytometry that over 99% of the ovarian cancer cells express ADAM10, ADAM17, and Nectin-4 on their surface, making co-localization likely for cleavage in the cis configuration. It has not yet been established whether LPA stimulation works by increasing the expression of ADAMs or by some other mechanism. Lorenzen et al. (54) recently showed that PMA stimulates shedding by ADAM17 and rapidly reduces most of the mature ADAM17 (but not its pro-form) through internalization. Although physiologic activation similarly stimulated shedding by ADAM17, the amount of mature ADAM17 was unchanged; however, this effect has not been observed for ADAM10 (54). Because ADAM proteases play a role in many physiological and pathophysiological pathways, they must be tightly regulated. This is achieved in part by storing most of the active protease intracellularly, whereas only smaller amounts are available on the cell surface. Because ADAM17 and ADAM10 are closely related and share many substrates, we assume that ADAM10 activity is similarly tightly regulated, especially with physiological stimulation such as LPA.

Knockdown of ADAM17 and ADAM10 using siRNA was not sufficient to block Nectin-4 shedding completely in our study. This is likely due to the presence of ADAM protein synthesized prior to siRNA treatment, because adding the double inhibitor INCB3619 to the combined siRNA knockdown cells, blocking activity of the residual ADAM proteases, led to over 93% inhibition of Nectin-4 shedding.

We further showed that ascites fluid from high-grade serous ovarian cancer patients caused Nectin-4 to be shed. We hypothesize that this might be due to LPA in ascites fluid, although we have not quantified the LPA content in our samples. The composition of ascites is complex and provides a beneficial pro-inflammatory microenvironment for tumor cells in the hypoxic milieu of the ascites fluid (55). Ascites contains many cell types, growth factors such as VEGF and EGF, and cytokines such as IL-6 and IL-8. A recent study examined proteins from intratumor fluid of high-grade serous ovarian cancer tumors versus benign serous cystadenomas (55). Over 75% of the proteins identified were detected in the malignant tumor fluid, but not in the benign group. Of relevance to our study are their findings showing that: (a) malignant tumors secrete a complex mixture of proteins (which would likely be present in the ascites fluid), (b) LPA may not be the only molecule that stimulates proteases, and (c) adhesion is reported to be one of the major pathways involved in the functional activity of the identified proteins.

To assess our hypothesis that LPA in ascites fluid stimulates Nectin-4 shedding, we used the LPA receptor antagonist Ki16425, which blocks the human LPA1, LPA2, and LPA3 receptors (56). In our experiments, Ki16425 did not significantly block shedding of Nectin-4 after stimulation of ovarian cancer cells with ascites fluid (data not shown). Because of the complex composition of ascites fluid, a combination of several activating factors may induce Nectin-4 shedding, especially because heterogeneous stimuli are known for ADAM proteases. Thus, it is unlikely that LPA is the sole responsible factor.

Our data showed that ovarian cancer patients expressed Nectin-4, ADAM10, and ADAM17 mRNA in their primary tumor, as well as in cells isolated from their ascites fluid. Omental tumor tissue showed a more varied expression of the three genes among the patients, which is consistent with the clonal nature of ovarian cancer metastasis (57). Although the sample size prevents a rigorous comparison, two of the patients (patients 6 and 11) were also tested for sN4 levels in serum and ascites fluid. It is interesting to note that in these patients, the level of sN4 in serum and ascites correlated with the level of Nectin-4 mRNA expression in the primary tumor.

Our preliminary signaling pathway screen shows a major role for Nectin-4 in the LPA stimulation of AKT signaling. AKT can be activated by treatment with LPA (58) and by ascites (59), and patients with AKT2 alterations have a poor prognosis (60). AKT promotes EMT (61), which corroborates our recent work in which Nectin-4 knockdown cells had increased expression of mesenchymal markers such as vimentin and decreased expression of epithelial markers such as E-cadherin (15).

Other factors substantially altered in our screening assay include EGFR, ERK1/2, JNK1/2/3, and c-Jun. Both ADAMs promote cancer progression through the release of EGFR ligands, which activates signaling through AKT and ERK (53, 62). Overexpression of EGFR is associated with a poor prognosis (63), but treatment with EGFR inhibitors has led to low clinical response rates (64). Our study suggests that by targeting Nectin-4 in ovarian cancer, phosphorylation of EGFR, AKT, and ERK can be reduced, which might be a promising therapeutic strategy.

Fig. 7 shows our model of the potential mechanism of Nectin-4 shedding in ovarian cancer progression. As depicted in Fig. 7a, the primary ovarian tumor expresses Nectin-4, ADAM17, and ADAM10. Both ADAMs play a role in Nectin-4 shedding from the surface of cancer cells as shown in this study (Fig. 7b). The shed ectodomain of Nectin-4 can be detected in ascites and serum samples (Fig. 7c) of ovarian cancer patients (this study and Ref. 9) and might have a signaling function or could potentially act as a negative regulator of cell adhesion.
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Figure 7. Model for ovarian cancer progression in which Nectin-4 plays a critical role. a, ovarian cancer cells (green solid ovals) express Nectin-4 (purple four), ADAM10 (blue rod), and ADAM17 (orange rod) on their surface (Refs. 8, 9, and 15 and this study). b, the ectodomain of Nectin-4 can be cleaved from the surface of ovarian cancer cells by ADAM10 and ADAM17 (this study). c, shed Nectin-4 can be detected in ascites fluid of ovarian cancer patients (this study) and in the serum of ovarian cancer patients (Ref. 9 and this study). d, Nectin-4 expressing ascites cells can be found in patients (this study) and Nectin-4 is essential for the formation of compact spheroids (15). e, ovarian cancer spheroids adhere to Nectin-1 (blue ones) on mesothelial cells (blue rectangles) (4, 15). f, ovarian cancer spheroids can then disaggregate and invade mesothelial cell layers (5, 6). g, the presence of Nectin-4, ADAM10, and ADAM17 can be detected at secondary tumor sites (omentum tissue in this study). Our data from this study and previous work implies that ovarian cancer cells strongly rely upon Nectin-4-mediated cell-cell interactions within the peritoneal cavity to metastasize.

Ovarian cancer cells exist both as free-floating single cells and as aggregated spheroids in ascites fluid, and our recent study showed that Nectin-4 is essential for the formation of compact spheroids (Fig. 7d) (15). We have previously published that spheroids adhere to mesothelial cells (Fig. 7e) (4–6), possibly through binding to Nectin-1 (15), and then invade the mesothelial cell layer (Fig. 7f), potentially through the cleavage of Nectin-4, thereby promoting migration and tissue invasion and eventually formation of a secondary tumor (Fig. 7g).

The results of this study and our previous work support the role of ADAM proteases and Nectin-4 cleavage in ovarian cancer progression. We have observed that cells expressing Nectin-4 migrate faster than Nectin-4 knockdown cells in a scratch wound assay (15). ADAM10 has been shown to be involved in the migration of cells, either in conjunction with ADAM17 through ectodomain cleavage of the adhesion molecule CD44 (23) or alone for leukocyte migration to the alveolar space (65). Furthermore, ADAM10 was recently shown to play a role in breast cancer progression, although their study did not look at shedding of Nectin-4 (26). In a mouse model of ovarian cancer, treatment with the specific anti-ADAM17 antibody D1(A12) showed a modest anti-tumor effect; however, circulating TNF-α levels remained the same. The authors suggested that ADAM17 inhibition was compensated for by another enzyme (35), which is likely ADAM10. A recent study on a Nectin-4 antibody conjugated to a microtubule-disrupting agent reported tumor regression using xenograft models of bladder, breast, pancreatic, and lung cancer, validating Nectin-4 as a target in solid tumors (7). The results of this study, in combination with our previous work (9), provide further justification for the use of Nectin-4 as a biomarker for ovarian cancer, whereas the combined targeting of Nectin-4, ADAM10, and ADAM17 might be a promising target for ovarian cancer therapy.

Experimental procedures

Cell lines

The human NIH:OVCAR5 cell line, which was recently characterized as a good representative for high-grade serous ovarian cancer experimental models (66), was used in this study. The cell line was received from Judah Folkman (Harvard University) and viably stored in liquid nitrogen. The cells were grown in complete medium (RPMI 1640 medium containing 10% FBS) at 37 °C in a humidified incubator with 5% CO2. Cell lines were verified by short tandem repeat fingerprinting (M. D. Anderson Characterized Cell Line Core Facility, Houston, TX).

The NIH:OVCAR5 cell line, which overexpresses Nectin-4 (NIH:OVCAR5-N4-over), was generated by transfection of a full-length Nectin-4 cDNA cloned into the p3XFLAG-myc-CMV-25 expression vector (Sigma-Aldrich) kindly provided by Dr. Marc Lopez (Centre de Recherché en Cancérologie de Marseille, Marseille, France) as previously described (15). FACS was used to select for stably transfected cells that expressed high levels of Nectin-4 compared with the parental cell line (15).

NIH:OVCAR5 cells were transfected with either an shRNA targeting Nectin-4, or a control shRNA, as previously described (15). Nectin-4 expression was knocked down by 85% in clone VB3 (hereafter termed NIH:OVCAR5-N4-KD). The corresponding NIH:OVCAR5 shRNA control cells expressed endogenous levels of Nectin-4.

Biospecimens

Samples from patients diagnosed with stage III or IV serous ovarian carcinoma were obtained through the University of Minnesota Tissue Procurement Facility with approval of the University of Minnesota Institutional Review Board. Blood was collected immediately before surgery from women with an abdominal mass suspected to be ovarian cancer, and ascites fluid was collected during the surgical procedure. Samples were processed by standard operating procedures (4) and divided into aliquots. Serum and ascites fluid were stored at −80 °C. Snap-frozen tumor tissues and cryopreserved ascites cells were stored in liquid nitrogen. Before use, the pathology reports were reviewed by a gynecologic pathologist to ensure that the samples were high-grade serous ovarian cancer. RNA of the ascites cells, primary tumor, or the omentum tumor tissues was extracted using the Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s instructions, and the samples were analyzed by RT-PCR using the primers in Table 2.
Flow cytometry

Confluent monolayers of cells were detached from 75-cm² tissue culture flasks using Accutase cell dissociation buffer (Innovative Cell Technologies, San Diego, CA), washed, and labeled with mouse IgG or antibodies against Nectin-4, ADAM10, or ADAM17 (2.5 μg/10⁶ cells) in flow buffer (PBS containing 2.5% newborn calf serum and 0.02% sodium azide) for 30 min at 4 °C (9). The cells were washed and incubated with goat anti-mouse IgG F(ab')₂ (Jackson ImmunoResearch, West Grove, PA), then washed again, and incubated with streptavidin-allophycocyanin conjugate (Jackson ImmunoResearch) for 30 min each. The cells were washed and fixed in flow buffer containing 1% formaldehyde and analyzed in the University of Minnesota Flow Cytometry Shared Resource with a BD Accuri™ C6 flow cytometer (BD Biosciences) using the Accuri™ software (for Nectin-4 staining) or a BD LSR II flow cytometer using FlowJo software (FlowJo, Ashland, OR) for ADAM10 and ADAM17 staining.

Nectin-4 ectodomain shedding assay

NIH/OVCAR5-N4-over cells were seeded in 24-well plates at 6 × 10⁴ cells/well. After 24 h in culture, the cells were washed once with PBS, and then 400 μl of OptiMem (Gibco, Life Technologies) was added. The broad-spectrum and selective ADAM sheddiing inhibitors were used as previously described (25, 34, 67) (Table 1). The appropriate stimulus was added to each well, and the plates were incubated at 37 °C for varied times. The pleiotropic stimulator PMA was used at 100 ng/ml (Sigma-Aldrich), and the physiologic stimulator oleoyl-1-α-lysophosphatidic acid sodium salt (LPA) was used at 10 μM (Cayman Chemical, Ann Arbor, MI). The supernatant was subsequently carefully transferred to a microtube and centrifuged for 4 min at 400 × g, and supernatants were stored either at 4 °C overnight or at −20 °C, if samples were not processed the next day. Shed Nectin-4 was quantified using the human Nectin-4 DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions. The plates were read in a microplate reader (BioTek, Winooski, VT). To inhibit ectodomain shedding of Nectin-4, inhibitors (Table 1) were added to the wells for 30 min at room temperature prior to stimulation with PMA or LPA. The unpaired Student’s t test was used to calculate significant inhibition of shedding by the inhibitors used (**, p < 0.01; *, p < 0.05); each sample with inhibitor was compared against the corresponding untreated control sample.

siRNA-mediated knockdown of ADAM proteases

On-Targetplus siRNA oligonucleotides against ADAM10 (SMARTpool; accession no. NM_001110) and ADAM17 (SMARTpool; accession no. NM_003183) were purchased from Dharmacon (Lafayette, CO). An On-Targetplus GAPDH control pool (accession no. NM_002046) and a non-targeting siRNA pool (catalog no. D-001810-10-05) were used as controls. For knockdown of ADAM proteases, 3 × 10⁴ cells were seeded in 24-well plates in antibiotic-free RPMI supplemented with 10% FBS (full RPMI). After incubation overnight, 25 nM of siRNA was mixed with 1.2 μl of DharmaFECT 1 transfection reagent in OptiMem according to the manufacturer’s instructions. For the simultaneous knockdown of ADAM17 and ADAM10, 25 nM of each siRNA was used. In addition to the negative control pool and the GAPDH-positive control, an untreated control without siRNA transfection was prepared. The cells were washed once with PBS, and 500 μl of full RPMI containing the siRNA was added to each well. After 24 h of incubation, the cells were washed once with PBS, and new full RPMI medium was added to each well. The following day, inhibitors of ADAM proteases were added, and wells were stimulated with PMA or LPA as described above. Supernatants were collected for quantification of sN4 by ELISA, and shedding in percentages was quantified relative to the shedding observed with the negative control siRNA pool. The unpaired
Student’s t test was used to calculate significant inhibition of shedding (**, p < 0.01; *, p < 0.05) (each knockdown sample was compared against the GAPDH control and the knockdown sample with inhibitor). From the remaining cells, total cellular RNA was extracted using the RNeasy micro kit (Qiagen) according to the manufacturer’s instructions, and samples were analyzed by duplex RT-PCR (for GAPDH plus ADAM10 or ADAM17) and agarose gel electrophoresis.

**RT-PCR**

50 ng of total RNA was amplified using the Access RT-PCR system (Promega Corporation, Madison, WI), which uses AMV reverse transcriptase for first strand cDNA synthesis and Tfl DNA polymerase for subsequent DNA amplification with gene specific primers (Table 2). Amplification products were visualized on a 0.9% agarose gel stained with SYBR® Gold (Invitrogen), and pictures were documented with a FluorChem E system (ProteinSimple, San Jose, CA). Densitometry was performed using ImageJ (National Institutes of Health, Bethesda, MD) (68).

**Quantification of shed Nectin-4 in patient serum and ascites samples**

Nectin-4 was quantified using the human Nectin-4 Quantikine ELISA kit (R&D Systems) according to the manufacturer’s instructions. Serum samples were diluted 1:5, and ascites samples were diluted 1:20 in OptiMem before testing.

**Stimulation of NIH:OVCAR5-N4-over cells with ascites fluid from high-grade serous ovarian cancer patients**

The cells were seeded as for the Nectin-4 ectodomain shedding assay (see above). The cells were washed once with PBS, and 400 μl of ascites fluid (diluted 1:10 in OptiMem) was added to the wells and incubated for 3 h at 37°C. For inhibition of Nectin-4 shedding, the dual ADAM10/17 inhibitor INCB3619 was diluted in 200 μl of OptiMem and incubated for 30 min at room temperature, before adding 200 μl of ascites sample (diluted 1:5). To determine the level of sN4 already present in patient ascites, diluted ascites samples were incubated in wells without cells. The supernatants were carefully removed and stored at −20°C. Nectin-4 was quantified using the human Nectin-4 DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions. The unpaired Student’s t test was used to calculate significant stimulation of shedding by incubation in ascites fluid (Fig. 5B, white triangles) or significant inhibition of shedding with INCB3619 (Fig. 5B, white squares) (**, p < 0.01; *, p < 0.05).

**Proteome profiler arrays**

NIH:OVCAR5 cells (N4-KD and control) were analyzed for potential signaling pathways using Proteome Profiler™ human phospho-RTK and human phosphokinase antibody arrays (R&D Systems). The cells were seeded in T75 flasks, grown to ~50% confluency, then serum-starved overnight, and stimulated with 10 μM LPA for 1 h. After LPA treatment, the supernatant from stimulated and unstimulated cells was collected, and Nectin-4 shedding was verified by ELISA as described above for the shedding assay. Protein extracts were prepared and incubated with antibody arrays according to the manufacturer’s instructions for each array type. Images were documented with a FluorChem E system (ProteinSimple), and spot densitometry was performed using ImageJ (68).

**Ectodomain shedding of Nectin-4 in ovarian cancer**

**Author contributions**—P. C. B. conducted most of the experiments, analyzed the results, prepared figures, and wrote most of the paper. K. L. M. B. provided technical assistance, provided input for design of the experiments, conducted the signaling pathway screen and its analysis, and helped with writing the paper. R. H. conducted the PCR experiment for the matched patient biospecimens. B. W. provided some of the inhibitors, provided technical assistance for the experimental conditions, and helped with the design of the paper. M. A. G. and P. A. A. provided input on patient consenting, biospecimen collection, and review of pathology reports. A. P. N. S. conceived the idea for the project, oversaw the experimental design, and helped write the paper. All authors reviewed the results and approved the final version of the paper.

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