The Effect of Disintegrin–Metalloproteinase ADAM9 in Gastric Cancer Progression

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Abstract

Advanced gastric cancer is one of the most aggressive gastrointestinal malignancies, and ADAM (A disintegrin and metalloproteinase)-9 is a cell-surface membrane glycoprotein with oncogenic properties that is overexpressed in several cancers. Herein, we investigated the biologic mechanism of ADAM9 in the progression, proliferation, and invasion of gastric cancer. First, we detected ADAM's expression, processing, and protease activity in gastric cancer cells. Protease activity was moderately correlated with ADAM9 protein expression, but was better related to a processed smaller molecular weight (84 kDa) form of ADAM9. Knockdown of ADAM9 or specifically targeted monoclonal antibody (RAV-18) suppressed cancer cell proliferation and invasion in high ADAM9-expressing cells, not in low ADAM9-expressing cells. RAV-18 showed in vivo antitumor activity in a gastric cancer xenograft model. Hypoxia (1% oxygen) induced ADAM9 expression and functional activity in low ADAM9-expressing gastric cancer cells that was inhibited by siRNA knockdown or RAV-18 antibody to levels in normoxic cells. Overall, our studies show that ADAM9 plays an important role in gastric cancer proliferation and invasion, and that while expressed in some gastric cancer cells at high levels that are responsive to functional inhibition and antitumor activity of a catalytic site–directed antibody, other gastric cancer cells have low levels of expression and only when exposed to hypoxia do ADAM9 levels increase and the cells become responsive to ADAM9 antibody inhibition. Therefore, our findings suggest that ADAM9 could be an effective therapeutic target for advanced gastric cancer.

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Introduction

Gastric cancer is the second leading cause of cancer-related mortality worldwide with a median survival of only around a year after diagnosis of advanced disease. This dismal prognosis mainly results from early invasion and high metastatic activity with an unknown molecular basis. Cancer metastasis is a complex, multistep process involving the interplay between cancer cells and surrounding components of extracellular matrix (ECM), and “invasion” is the first step by which cancer cells break away from the primary tumor and infiltrate surrounding stoma (1). Proteolysis has emerged as a key posttranslational regulator of invasion in gastric cancer (2, 3). However, the mechanism and major players in invasion have not yet been fully elucidated.

A disintegrin and metalloproteases (ADAM) are modular type I transmembrane proteins that contain a metalloprotease and a disintegrin-like domain, and recent studies have demonstrated a relationship between increased ADAMs and cancer progression (4). Twenty-one members of ADAM family have been identified in humans, 13 of which have functional proteases. ADAM9 is shown to possess potent biologic activities and is highly expressed in cancers, including prostate, pancreas, breast, and colon, associated with cancer progression and poor clinical outcome (5–9). In prostate cancer, microenvironmental stress on tumor cells leads to shedding of proheparin-binding epidermal growth factor (EGF), which is processed by ADAM9. The metalloprotease domain of ADAM9 also cleaves the insulin β-chain, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-α, gelatin, β-casein, and so on, and induces the shedding of EGF, fibroblast-growth factor receptor...
2IIIB, and heparin-binding EGF-like growth factor (10, 11). Therefore, the abilities of ADAM9 to degrade specific ECM substrates, release active growth factors, interact with key regulatory factors, and its expression at the invasion edges of several cancer metastases suggest that ADAM9 regulates several cancer-related processes—including cell growth and invasion.

A previous report has shown that the transcription of ADAM9 is upregulated in gastric cancer (12); however, its biologic function and potential as a therapeutic target are largely unknown. In this study, we investigated the biologic role of ADAM9 in gastric cancer invasion, and explored a therapeutic approach of antimetastasis treatment for high ADAM9-expressing cancers using an ADAM9-specific inhibitory antibody.

Materials and Methods

Reagents and antibodies

Matrigel was supplied by BD Biosciences. Recombinant ADAM9 protein was from R&D Systems Inc. ADAM9 antibodies were from Cell Signaling Technology for immunoblot and GeneTex for immunohistochemistry. Antibodies of hypoxia-inducible factor-1α (HIF1α) and total/phospho forms of EGFR and ERK were obtained from Santa Cruz Biotechnology. Antibodies against GAPDH and α-tubulin were purchased from Abcam and Sigma-Aldrich. Total and phospho-Akt antibodies were purchased from Cell Signaling Technology. RAV-18, an anti-ADAM9-specific blocking antibody, was kindly provided by Macrogen Inc.

Cell culture

The human gastric cancer cell lines AGS, NCI-N87, Hs746T, and KATO-III and the cervical cancer cell line HeLa, positive control of ADAM9, were purchased from the American Type Culture Collection (ATCC) in 2012. SNU-1, -5, and -16 in 2006, SNU-216, -638, and -668 in 2007, SNU-484 in 2008, MKN-1, -28, -45, and -74 were supplied from the Korean Cell Line Bank (KCLB). Above cell lines were authenticated by standard short tandem repeat (STR) DNA typing methodology before being purchased from the ATCC and KCLB. YCC-1, -2, -3, were established in 1994, and YCC-9, -10, -11, and -16 were established in 2000 by the Cancer Metastasis Research Center at Yonsei University College of Medicine (Seoul, Korea) from the ascites or peripheral blood (YCC-16) of patients with advanced gastric cancer. YCC cell lines were authenticated by STR in July 20, 2012. All cells were maintained in RPMI-1640 (Nissui) and supplemented with 10% FBS (Omega Scientific, Inc.), 100 U/mL ampicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (Gibco) in 5% CO2 humidified atmosphere at 37°C. All cell lines were expanded and cryopreserved in liquid nitrogen in our laboratory.

RNA extraction and RT-PCR

Total RNA was extracted using TRizol reagent (Qiagen Science) according to the manufacturer’s protocol. Briefly, 500 µL of TRizol was added to 1 × 10⁷ cells, and after adding 200 µL chloroform, cell homogenates were centrifuged at 13,200 rpm for 25 minutes at 4°C, and the aqueous phases were collected. Total RNA was precipitated with the same volume of isopropanol for 60 minutes at −20°C and centrifuged for 30 minutes. The pellets were washed once with 70% ethanol and suspended in RNase-free water. The quantity of total RNA was measured using a NanoDrop spectrophotometer (Thermo Scientific).

To synthesize the cDNA, 4 µg of total RNA from each cell line was mixed with the oligo-dT primer and incubated at 65°C for 10 minutes. After adding the Super-Script II, 5× first strand buffer, 100 mmol/L DTT, and 10 mmol/L dNTP mix to the RNA/oligo-dT mixture, a reverse transcription process was performed at 42°C for 90 minutes. The remaining RNA was hydrolyzed by incubation at 65°C for 15 minutes in 0.1 N NaOH. The reaction was then neutralized by the addition of an equal volume of 0.1 N HCl.

Amplification reactions were performed in an Eppendorf Mastercycler Gradient (Eppendorf). PCR was performed using 1 µg of cDNA, 2.5 mmol/L dNTPs, 1.5 mmol/L 10× PCR buffer with MgCl₂, and U Taq polymerase (Invitrogen) to total 50 µL of reaction volume. The primer sequences used were as follows: ADAM9 (forward: 5'-AGT GGC GGG AAA AGT TTC TT-3'; reverse: 5'-CCA GCG TCC ACC AAC TTA TT-3'). The conditions for PCR amplification were as follows: 94°C for 2 minutes followed by 94°C for 40 seconds, 60°C for 50 seconds and 72°C for 1 minute, then 72°C for 2 minutes, and repeated for 30 cycles. PCR products were separated on ethidium bromide gels containing 1.2% agarose.

Exposure to hypoxic conditions

When cells reached 80% confluence in a 25 cm² flask, the flask was placed in a hypoxic chamber (Personal O₂/CO₂ Incubator; Aspect). Cells were exposed to 1% O₂, 5% CO₂, and 37°C for 24 hours. After that, cells were collected and transferred to a Matrigel-coated 24-well plate for invasion assay. Invaded cells were stained using 0.5% crystal violet and counted after 24 hours. Protease activity assay, immunoblotting, and proliferation assay were conducted using cells exposed to hypoxic conditions in the same way.

Immunoblotting

Subconfluent cells were harvested and suspended in a whole cell lysis buffer [50 mmol/L Tris (pH 8.0), 10% glycerol, 2 mmol/L EDTA, 5 mmol/L NaF, 1% NP-40, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 10 µg/mL aprotonin, 10 µg/mL leupeptin, and 1 mmol/L phenyl-methyl-sulfonyl fluoride]. The lysates were subjected to SDS-PAGE and blotted onto the polyvinylidenedifluoride (PVDF) membrane. The
membrane was then incubated with the appropriately diluted primary antibody overnight at 4°C, followed by a horseradish peroxidase (HRP)–conjugated secondary antibody for 60 minutes at room temperature. The membrane was visualized with an ECL Western blotting reagent kit (Amersham Bioscience).

**Measurement of ADAM9 protease activity**

Protease activity of ADAM9 was measured by a modification of the method of Cisse and colleagues (13). Briefly, 5 \( \times 10^6 \) cells were seeded in each well of a 96-well plate (BD Falcon) and cultured for 1 day. The cells were rinsed with PBS twice, and 100 μL PBS containing 1 μmol/L ADAM9 substrate (R&D Systems Inc.) was added and incubated at 37°C for 8 hours. Supernatant was transferred to a black 96-well plate and the fluorescence activity was measured at 320/405 nm using a Spectrophotometer (PerkinElmer). To normalize the fluorescent activity to cell number, the cells remained in the plate and were treated with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) solution. After incubation for 4 hours, the MTT solution was discarded and the MTT-formazan crystals were dissolved in 150-μL DMSO. The absorbance was measured at 570 nm using a multwell ELISA automatic Spectrometer (Behring ELISA Processor II). The protease activity was calculated using the following equation: protease activity \( = \) (fluorescence at 320/405 nm)/absorbance of MTT.

**ADAM and HIF1α knockdown experiment**

Small interfering RNA (siRNA) against ADAM9 was purchased from Origene and was also independently designed using the Genescript siRNA Finder (https://www.genscript.com/ssl-bin/app/rnai). HIF1α siRNA was designed by Genescript siRNA Finder. The ADAM9 and HIF1α siRNA target sequence are as follows: ADAM9, 5′-GACTGTCGGTTCCTTCCAG-3′ and HIF1α, 5′-GTCGTCAACATGGAAGGTA-3′. ADAM-10 and -17 siRNA were supplied from Bioneer Corporation. Cells were transfected with Lipofectamine MAX (Invitrogen) by the following equation: DT = \( \frac{\text{time after cell growth \times time before cell growth}}{\log_2 \text{of number of cells after growth}} \).

**Matrigel Transwell assay**

The lower side of an insert (diameter, 6.5 mm; pore size, 8 μm; Becton Dickinson) was coated with 8 μg/μL Matrigel. Filters were placed on a 24-well containing media supplemented with 0.1% bovine serum albumin (BSA). Cells were harvested with a cell dissociation medium alone (without the drug). Cells (5 \( \times 10^6 \)) were seeded in a 24-well plate with or without treatment of RAV-18. Cells were counted every day for 7 days. Cellular doubling time (DT) was calculated by the following equation: DT = \( \frac{\text{time after cell growth} - \text{time before cell growth}}{\log_2 \text{number of cells after growth}} \).

**In vivo xenograft model**

BALB/c nu/nu mice (female, 7-weeks old; SLC Inc.) were housed under specific pathogen-free conditions. Experiments were performed according to the standard guidelines for animal experiments of Yonsei University College of Medicine. The effect of RAV-18 on the xenograft model was examined as follows: 1 \( \times 10^7 \) MKN-28 cells were inoculated subcutaneously (s.c.) in the flank of the mouse or injected intraperitoneally (IP). The mice were divided into four groups: a control group of s.c. (PBS i.p.; \( n = 7 \)), a RAV-18–treated group of s.c. (50 mg/kg i.p., five times for 2 weeks; \( n = 7 \)), a control group of IP (PBS i.p.; \( n = 7 \)), and a RAV-18–treated group of IP (50 mg/kg i.p., five times for 2 weeks; \( n = 7 \)). The treatment was started on day 21 after cell inoculation and mice were sacrificed after 8 weeks. Tumor
volume and body weight were measured twice weekly. The tumor volume was calculated using the formula: volume = length × width × width × 0.5. At the end of the experiment, tumors and peritoneal nodules were collected. The weights of collected samples were measured and the peritoneal nodules were counted.

Immunohistochemistry

Tumor specimens were fixed in 10% formaldehyde and embedded in paraffin. All samples were cut into 5-μm-thick sections for immunohistochemistry (IHC). The sections were stained with H&E and immunostained with anti-ADAM9 (1:100), anti-pEGFR (1:200), and anti-pERK (1:100) antibodies at room temperature for 90 minutes. The sections were reacted with an EnVision reagent (Dako Co.) for visualization. The results of immunostaining were categorized as follows: staining in less than 10% of the tumor cells was scored as 0; staining in more than 10% of the tumor cells was scored as 1+; weak to moderate staining in more than 10% of the tumor cells was scored as 2+; and strong staining in more than 10% of the tumor cells was scored as 3+.

Statistical analysis

Quantitative data were represented as the mean ± standard deviation (SD) of at least three independent experiments. Statistical comparison between groups was done using the Student t test. Differences were regarded as statistically significant when the P < 0.05.

Results

Screening of ADAM9 expression and protease activity in the gastric cancer cell panel

We first carried out immunohistochemistry on paraffin-embedded tumor sections from 10 patients with gastric cancer with tumor infiltrating beyond subserosa (≥T3). Four of 10 (40%) cancers expressed ADAM9, whereas no expression was found in adjacent noncancerous tissue. ADAM9 expression was highest in cells along infiltrating margins bordering noncancerous epithelium, and was located at the membrane and in the cytoplasm (Fig. 1A).

Then, we screened for ADAM9 expression in 24 gastric cancer cell lines. The mRNA levels varied (Supplementary Fig. S1) and did not correlate with ADAM9-protein expression in the cell lines (R² = 0.106; P = 0.203). In immunoblots, ADAM9 was detected as two bands: a proform (110 kDa) and mature form (84 kDa; Fig. 1B; ref. 14). For further experiments, we selected four cells with high ADAM9 protein expression and protease activity (SNU-638, YCC-1, MKN-74, and MKN-28) and three cells with low ADAM9 protein expression and protease activity (YCC-6, YCC-7, and Hs746T) as being outside of 1-SD of the mean value of protease activity tested (Fig. 1C). Protease activity and protein expression of the mature form were correlated each other (Fig. 1D; R² = 0.493; P < 0.01).

Effect of ADAM9 suppression in gastric cancer cells

First, we screened four siRNA against ADAM9 and selected the most efficient one for further experiment (Supplementary Fig. S2). In cells with high ADAM9 expression, protein levels were reduced, and its protease activity was also decreased significantly by 61.8% ± 3.9% in SNU-638 and 42.7% ± 4.5% in MKN-28 (Fig. 2A and B; P < 0.01). The knockdown of ADAM9 led to decline in phospho-EGFR and phospho-ERK (Fig. 2A), Then, we examined the invasion after siADAM9 transfection. Cells transfected with siADAM9 showed a reduced invasion activity through Matrigel compared with control cells by 71.4% ± 2.2% in SNU-638 and by 53.0% ± 3.9% in MKN-28 (Fig. 2C; P < 0.01), which suggests that the decreased invasion may be a consequence of inhibited EGFR/ERK signaling.

Effect on the proliferation and invasion by inhibiting protease activity of ADAM9

ADAM9 is a proteolytically active enzyme and the protease activity is currently the well-defined function of ADAMs, with most of the putative substrates being transmembrane proteins. Therefore, we focused on the proteolytic domain of ADAM9 as a biomarker and therapeutic target using RAV18, an ADAM9-specific blocking antibody, for our studies. Gastric cancer cells with high expression of ADAM9 treated with RAV-18 showed a dose-dependent decrease in ADAM9 protease activity (Fig. 3A; 68.7% ± 0.8% in SNU-638, 62.1% ± 2.6% in MKN-28, and 50.3% ± 2.1% in MKN-74 at 20 μg/mL RAV-18; P < 0.05); however, there was no decrease in ADAM9 protease activity in cells with low expression of ADAM9.

Next, we investigated the effect of RAV-18 on cell proliferation. For SNU-638 cells, whose baseline doubling time was 22.6 ± 0.1 hours, after treatment with 20 μg/mL RAV-18, doubling time was prolonged to 32.3 ± 0.5 hours (P < 0.01). Similarly, proliferation was significantly delayed with other cells (YCC-1: 25.4 ± 0.2 vs. 33.9 ± 0.4 hours; MKN-28: 27.4 ± 0.1 vs. 36.3 ± 0.5 hours; MKN-74: 27.7 ± 0.2 vs. 39.3 ± 0.8 hours; P < 0.05). However, RAV18 did not affect proliferation in cells with low ADAM9 expression (Fig. 3B).

We investigated the effect of RAV-18 on cell adhesion. At a dose of 20 μg/mL RAV-18, adhesion to Matrigel was unchanged in any of cells tested (Supplementary Fig. S3). However, high ADAM9-expressing cells demonstrated reduced invasion by 60% in SNU-638, 47% in YCC-1, 31% in MKN-74, and 51% in MKN-28 (Fig. 3C; P < 0.05); however, there was no effect of RAV-18 in the low ADAM9-expressing cells. These findings can transfer that ADAM9 regulates cancer proliferation and invasion, which is dependent on its protease activity.

Phospho-EGFR and phospho-ERK were decreased in cells that responded to RAV18, which accords to the results of siADAM9 transfection. There was no effect of RAV-18 on phospho-EGFR and phospho-ERK signaling.

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in the low-ADAM9 cells that did not respond to RAV-18 (Fig. 3D).

**Effect of hypoxia on ADAM9 protease activity and cancer invasion**

Our hypothesis is that the effect of RAV-18 depends on its inhibition of ADAM9 proteolytic activity and if so, cells with low ADAM9 should respond to RAV-18 if ADAM9 is induced. We investigated the influence of hypoxia on the constitutively low ADAM9-expressing cells and found that exposure to 1% O₂ for 24 hours increased ADAM9 protease activity in YCC-6, YCC-7, and Hs746T cells, and this was returned to nonhypoxia levels by RAV-18 treatment in a dose-dependent manner (Fig. 4A).

We also found that the invasiveness of cells with low ADAM9 cells was increased under hypoxic conditions (by 24% in YCC-7 and 13% in Hs746T; P < 0.05). RAV-18 treatment significantly reduced the invasiveness in hypoxia (by 30% in YCC-7 and 14% in Hs746T at 20 μg/mL RAV-18; P < 0.05; Fig. 4B).

When these cells were incubated under hypoxic conditions, phospho- and total EGFR and ERK expressions were, as expected, induced. In addition, treatment of RAV-18 abrogated the EGFR and ERK phosphorylation (Fig. 4C). Taken together, these findings suggest that hypoxia, a feature present in many solid tumors, including gastric cancer, increases ADAM9 expression and activity, together with an increased invasion, that may contribute to the growth and spread of gastric cancer.

**Relationship with ADAM9 and hypoxia**

To obtain further information on the relationship between ADAM9 and hypoxia, we treated low ADAM9 cells with HIF1α siRNA under hypoxic condition. ADAM9 protease activity was significantly decreased
when HIF1α was suppressed, and on double siRNA inhibition of HIF1α and ADAM9, there was an additional effect in suppressing ADAM9 activity to normoxic levels (Fig. 5A; \( P < 0.05 \)). Under hypoxic conditions, cell proliferation was also increased and this effect was prevented with the treatment RAV-18 and HIF1α siRNA (Fig. 5B).

In immunoblots, we detected that ADAM9 and HIF1α expression in the cells were increased under hypoxic condition. When HIF1α siRNA were transfected, ADAM9 expression and EGFR and ERK activity was decreased, while AKT activity was unchanged. On double inhibition of HIF1α and ADAM9, these effects were pronounced, while HIF1α expression was not different compared with HIF1α siRNA alone (Fig. 5C). Thus, hypoxia and HIF1α induction may be a factor that regulates the ADAM9 expression and its downstream signaling that lead to cancer progression.

**In vivo experiment of RAV-18 for therapeutic effect**

To investigate whether ADAM9 protease activity was related to cancer progression in vivo, we conducted a xenograft study using BALB/c nu/nu mice. Mice were inoculated s.c. with MKN-28 (high ADAM9 protease activity) cells. At 3 weeks after inoculating the cells, when the tumor volumes reached around 100 mm<sup>3</sup>, mice were split into two groups and treated with either saline or 50 mg/kg RAV-18 intraperitoneally for 2 weeks. The RAV-18–treated group showed suppressed tumor growth compared with the saline-treated group (mean tumor volume: 692 \( \pm \) 219 vs. 1,568 \( \pm \) 557 mm<sup>3</sup>; \( P < 0.01 \); Fig. 6A).

Subsequently, MKN-28 cells were inoculated intraperitoneally into mice. After 3 weeks, saline and RAV-18 were administered as above. At 5 weeks of treatment, tumor nodules were significantly smaller in the RAV-18 group compared with the control group; the average tumor nodules in RAV-18–treated group were 58.3 \( \pm \) 17.8, while those of the control were 96.3 \( \pm \) 19.6, indicating a 40% reduction of tumor formation (Supplementary Fig. S4; \( P = 0.0001 \)). Immunoblot showed a decrease of phospho-EGFR and phospho-ERK expression following RAV-18 treatment as had been seen in vitro studies. The average p-EGFR histoscore of the control
Figure 3. Changes in ADAM9 protease activities, invasion, and proliferation with treatment of RAV-18. A, when cells with high ADAM9 protease activity were treated with RAV-18, a dose-dependent decrease in protease activity was seen (from SNU-638 to MKN-28). However, cell lines with low ADAM9 protease activity showed no change in protease activities (from YCC-6 to Hs746T). B, cell lines with high ADAM9 activity treated with RAV-18 showed dose-dependent inhibition of proliferation. However, the proliferation of cell lines with low ADAM9 activity was not inhibited. C, RAV-18 also dose-dependently reduced invasiveness in cell lines with high ADAM9 activity, but did not influence invasiveness of low activity cells (*, $P < 0.05$). D, when cell lines were treated with RAV-18, pEGFR, and pERK, expression was dose-dependently decreased in cell lines expressing high ADAM9; low ADAM9-expressing cell lines did not demonstrate a change.
group was 188.1 ± 68.4, while that of the RAV-18 group was 98.6 ± 34.9 (P = 0.018). The average p-ERK histoscore was 139.3 ± 35.7 in the control group and 63.4 ± 21.2 in the RAV-18 group (P = 0.002; Fig. 6B and C).

These findings suggest that ADAM9 protease activity regulates gastric cancer tumorigenesis, including in a model for intraperitoneal tumor spread, and may be a therapeutic target in gastric cancer.

**Discussion**

In this study, we have demonstrated that ADAM9 is expressed in gastric cancer cells, which is related to cancer proliferation and invasion. In addition, using the protease domain-specific blocking antibody RAV-18, we showed that this effect depends on the protease activity of ADAM9. The level of the ADAM9 enzymatic activity in cells with low ADAM9 expression is induced by hypoxia, and inhibited by RAV-18. We further showed that ADAM9 protease activity regulates gastric cancer tumorigenesis, including intraperitoneal metastatic spread.

Taken together, these findings suggest that inhibition of ADAM9 activity is a rational therapeutic approach to suppress gastric cancer progression and metastasis.

There has been an earlier report demonstrating that ADAM9 transcription is increased in gastric cancer (12). Although little is known about the regulatory mechanism of ADAM9, some recent evidences suggest that a role for posttranslational modification is more important. In one prostate cancer model, induction of ADAM9 is regulated by stress through the accumulation of reactive oxygen species (ROS) as a common mediator, and removing ROS, or the use of antioxidants resulted in a marked reduction of ADAM9 (11). These findings suggest a link between stress-induced signaling and ADAM9, with ROS serving as a common mediator (15). We have shown that another stress factor, hypoxia, a common feature found in many solid tumors, leads to an increase in the expression of ADAM9 protein alongside enzymatic activity in HIF1-dependent way. We found that posttranslational modification and processing to a "mature" cleaved (84 kDa) form, is correlated...
with its enzymatic activity. Although all ADAMs possess the matrix metalloproteinase (MMP)–like domain adjacent to the prodomain, only 50% exhibit protease activity. Currently, protease activity is the best-defined function of ADAMs, with most putative substrates currently identified as being transmembrane proteins (16).

One of the limitations of our study is the substrate used for ADAM9 protease activity is not only specific to ADAM9, but also reactive to other ADAMs—especially ADAM10 and ADAM17 (17–21). In Supplementary Fig. S5, (i) changes of protease activity after siRNA knockdown is dependent on basal expression level of each ADAM, and (ii) when cotreated with RAV-18, additive effect is prominent only in high ADAM9-expressing cells, implying that RAV-18 works specifically to ADAM9.

Several explanations are possible about how ADAM9 plays a role in cancer progression. There is evidence that ADAM9 mediates the release of growth factor ligands, which regulate EGFR signaling. In the stomach, trans-activation of the EGFR by peptides, such as gastrin, angiotensin II, Bradykinin, bombesin, or substance P, is mediated by shedding of EGFR ligands by ADAMs (22, 23). ADAM9 also releases some transmembrane protein-derived ligands, such as heparin-binding EGF.
amphiregulin, TGF\(\alpha\), and TNF\(\alpha\) (24, 25). We herein found that ADAM9 knockdown blocks EGFR phosphorylation and downstream ERK activation, and that RAV-18 inhibited tumor growth, in line with EGFR and ERK phosphorylation in vivo. Thus, inhibition of EGFR ligand shedding by ADAM9 with subsequent decreased EGFR activation and downstream signaling could be a mechanism for the antitumor activity of RAV-18.

It is intriguing that hypoxia induces ADAM9 activity, relating to cancer cell proliferation and invasion, which is reversed by RAV-18 although not beyond the levels in normoxia. This does not appear to be related to the processing of ADAM9 to the cleaved form as this was detected in both low and high ADAM9-expressing cell lines. We speculate that there are two forms of ADAM9, one not responsive to inhibition by RAV-18, which is perhaps intracellular located or masked, and another induced by hypoxia that is accessible to inhibition by RAV-18, that is responsible for the growth stimulatory and invasive effects of ADAM9. Another point is that ADAM9 was induced by hypoxia in Hs746T cells but its downstream signaling was not altered by HIF\(\alpha\) knockdown or RAV-18 treatment. It is possible that Hs746T is more dependent on other ADAM proteins—such as ADAM10 and ADMA17—rather than ADAM9 for its biologic activity, as shown in Supplementary Fig. S2.

We have shown that proliferation and invasion is affected by RAV18, but not adhesion. The disintegrin domain is found in all ADAMs and is located downstream of the metalloproteinase domain. We believe that adhesion proteins are important for cellular adhesion, migration, and signal transduction (26). However, most information on the binding of disintegrin to integrin comes from in vitro studies using recombinant disintegrin domains (16). Thus, the biologic relevance of our finding that RAV-18 does not alter adhesion is unclear at this time.

Our in vivo studies demonstrate that ADAM9-targeting antibody is a reasonable option for treatment of gastric cancer. One limitation is lack of information on the actual incidence of ADAM9 expression in large-scale clinical samples. Our results suggest that only high-level
ADAM9 expressing tumors, and those where ADAM9 is induced in response to hypoxia, will be responsive to ADAM9 antibody. The subcellular location of ADAM9 could also play a role in the response to antibody treatment.

Collectively, our work shows that ADAM9 plays an important role in gastric cancer proliferation and invasion, and that while expressed at high levels in some cancer cells that are responsive to functional inhibition and antigrowth activity of a catalytic site-directed antibody, other gastric cancer cells have low levels of expression and only when exposed to hypoxia do ADAM9 levels increase and the cells become responsive to anti-ADAM9 treatment. These finding suggest that ADAM9 could be an effective therapeutic target for therapy of metastatic gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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