Chemotherapy-Induced Activation of ADAM-17: A Novel Mechanism of Drug Resistance in Colorectal Cancer

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Abstract

Purpose: We have shown previously that exposure to anticancer drugs can trigger the activation of human epidermal receptor survival pathways in colorectal cancer (CRC). In this study, we examined the role of ADAMs (a disintegrin and metalloproteinases) and soluble growth factors in this acute drug resistance mechanism.

Experimental Design: In vitro and in vivo models of CRC were assessed. ADAM-17 activity was measured using a fluorometric assay. Ligand shedding was assessed by ELISA or Western blotting. Apoptosis was assessed by flow cytometry and Western blotting.

Results: Chemotherapy (5-fluorouracil) treatment resulted in acute increases in transforming growth factor-α, amphiregulin, and heregulin ligand shedding in vitro and in vivo that correlated with significantly increased ADAM-17 activity. Small interfering RNA–mediated silencing and pharmacologic inhibition confirmed that ADAM-17 was the principal ADAM involved in this prosurvival response. Furthermore, overexpression of ADAM-17 significantly decreased the effect of chemotherapy on tumor growth and apoptosis. Mechanistically, we found that ADAM-17 not only regulated phosphorylation of human epidermal receptors but also increased the activity of a number of other growth factor receptors, such as insulin-like growth factor-I receptor and vascular endothelial growth factor receptor.

Conclusions: Chemotherapy acutely activates ADAM-17, which results in growth factor shedding, growth factor receptor activation, and drug resistance in CRC tumors. Thus, pharmacologic inhibition of ADAM-17 in conjunction with chemotherapy may have therapeutic potential for the treatment of CRC.

Resistance to chemotherapy is a major barrier in the treatment of cancer. Recent studies including our own have shown that exposure to anticancer drugs or ionizing radiation can activate stress pathways, which trigger activation of multiple signaling pathways, such as those regulated by the human epidermal receptor (HER) tyrosine kinase family (1–5).

The HER family of receptor tyrosine kinases (RTK) and their ligands are important regulators of tumor cell proliferation, angiogenesis, and metastasis (6–8). There are four receptors in the ErbB family: epidermal growth factor receptor (EGFR; HER1 or ErbB1), HER2 (neu or ErbB2), HER3 (ErbB3), and HER4 (ErbB4). HER1 is activated by binding of the HER1-specific ligands [epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and amphiregulin (AREG)] or ligands with dual specificity [heparin-binding EGF (HB-EGF), β-cellulin, and epiregulin (EREG)] to the ectodomain of HER1 (9, 10). HER1 and its ligand TGF-α constitute one of the best defined autocrine loops in human tumors (6, 11), and their coexpression correlates with aggressive disease and poor prognosis in several types of tumors, including colorectal cancer (CRC). Recently, high AREG and EREG mRNA expression levels in Kras wild-type colorectal primaries have been correlated with response and survival benefit following treatment with cetuximab and irinotecan in advanced CRC (12).

HER ligands are synthesized as transmembrane precursors that can be cleaved by cell surface proteases, particularly members of the ADAM (a disintegrin and metalloproteinase) family. ADAM-mediated ligand shedding results in enhanced juxtacrine and paracrine signaling (13). ADAMs are synthesized as inactive precursors containing a prodomain that blocks the activity of the catalytic domain. During transit through the secretory pathway, the prodomain of ADAMs is removed by...
Resistance to chemotherapy is a major barrier in the treatment of colorectal cancer (CRC). In this study, we show that cytotoxic chemotherapy treatment results in an acute increase in ADAM-17 (a disintegrin and metalloproteinase-17) activity in vitro and in vivo. Blocking ADAM-17 activity, using small interfering RNA or a small-molecule inhibitor, significantly increased apoptosis following chemotherapy treatment. We further show that overexpression of ADAM-17 increases activity of the human epidermal growth factor receptor and other prosurvival receptors, such as insulin-like growth factor-1 receptor and vascular endothelial growth factor receptor, and that this results in resistance to chemotherapy treatment in CRC tumors. Thus, targeting ADAM-17 in conjunction with existing chemotherapy treatments may enhance response rates in patients with advanced CRC by blocking the activity of multiple prosurvival receptors.

Materials and Methods

Materials

All chemicals and reagents of Analar grade were obtained from BDH Laboratory Supplies unless otherwise stated. GI254023X and GW280264X were provided by GlaxoSmithKline. A 10 mmol/L working solution of GI254023X and GW280264X in DMSO was prepared, aliquoted, and stored at -70°C. Oxaiplatin was obtained from Sanofi-Synthelabo. A 1 mmol/L stock solution was prepared in injection water and stored at 4°C. 5-Fluorouracil (5-FU) was purchased from Sigma Chemical Co. A 10 mmol/L stock solution in 1× PBS was prepared and stored at 4°C. SN-38 was obtained from Abatral, and a 2 mmol/L solution was prepared in DMSO and stored at 4°C.

Cells

All tissue culture material was obtained from Invitrogen unless otherwise stated. HCT116 and HCT116-p53null CRC cells were kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and maintained in McCoy’s 5A medium. LoVo CRC cells, supplied by AstraZeneca, were grown in DMEM. The RKO and H630 CRC cells were provided by the National Cancer Institute (Bethesda, MD) and maintained in DMEM. All medium was supplemented with 10% dialyzed FCS, 50 μg/mL penicillin-streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Invitrogen). All cells were grown in a humidified atmosphere with 5% CO₂ at 37°C.

Flow cytometric analysis and cell death measurement

Flow cytometry was done as previously described (5).

Annexin V analysis and apoptosis measurement

Cell pellets were resuspended in 100 μL of 1× binding buffer. Annexin V stain (5 μL) was added to each sample along with 5 μL of propidium iodide (PI) stain (50 μg/mL; 1:20 dilution in PBS of stock), and samples were incubated in the dark at room temperature for 15 minutes. After incubation, 320 μL of 1× binding buffer was added to each sample before analysis on the EPICS XL flow cytometer.

Western blotting

Western blot analysis was carried out as previously described (5). Immunodetections were done using anti-EGFR (clone 13; Pharmingen, BD Biosciences), anti-herégulin (R&D Systems), anti-AREG (R&D Systems), and anti-insulin-like growth factor (IGF)-I (Santa Cruz Biotechnology) mouse monoclonal antibodies in conjunction with a horseradish peroxidase–conjugated anti-mouse secondary antibody (Amersham). Anti-phospho-EGFR (Tyr1068; Calbiochem), anti–ADAM-17 (Pharmingen, BD Biosciences), anti-phospho-IGF-IR (Calbiochem), anti-phospho-vascular endothelial growth factor receptor 1 (VEGFR1; Calbiochem), anti–phospho-VEGFR2/3 (Calbiochem), and anti–platelet-derived growth factor receptor β (PDGFB; Calbiochem) rabbit polyclonal antibodies were used in conjunction with a horseradish peroxidase–conjugated anti-rabbit secondary antibody (Amersham). Equal loading was assessed using β-tubulin (Sigma) mouse monoclonal primary antibodies. The SuperSignal chemiluminescent system (Pierce) or ECL Plus (Amer- sham) was used for detection.

Small interfering RNA transfections

ADAM-9, ADAM-10, ADAM-12, ADAM-15, ADAM-17, and scramble control small interfering RNAs (siRNA) were obtained from Dharmacon. HCT116, HCT116-p53null, LoVo, RKO, and H630 CRC cells were seeded out in the...
appropriate media without penicillin-streptomycin. Twenty-four hours after seeding, siRNA transfections were done on subconfluent cells incubated in unsupplemented Opti-MEM using the Oligofectamine reagent (both from Invitrogen) according to the manufacturer’s instructions. After 4 hours, 3+ growth medium was added; cells were treated with oxaliplatin, 5-FU, or SN-38 1 hour following this.

Generation of stable hemagglutinin-tagged ADAM-17–overexpressing cells

The plasmid encoding the hemagglutinin (HA)-tagged full-length mouse ADAM-17 (HA-ADAM-17) was obtained as a kind gift from Dr. Atanasio Pandiella (Instituto de Microbiología Bioquímica and Centro de Investigación del Cáncer) and has been previously described (23). The pcDNA 3.1 empty vector (EV) was purchased from Invitrogen. HCT116 cells were cotransfected with 10 μg of HA-ADAM-17 construct or pcDNA 3.1 (EV) and 1 μg of a construct expressing a puromycin resistance gene (pIRE-Spuro3; Clontech) using GeneJuice transfection reagent (Novagen). Stably transfected cells were selected and maintained in medium supplemented with 1 μg/mL puromycin (Life Technologies, Inc.).

In vivo experiments

Female BALB/c severe combined immunodeficient mice were maintained under sterile and controlled environmental conditions (22°C, 50 ± 10% relative humidity, 12-h/12-h light/dark cycle, autoclaved bedding), with food and water ad libitum. Following 14 days of quarantine, mice were included in our protocol. The experiment was carried out in accordance with the Animals (Scientific Procedures) Act, 1986. To determine tumor volume, two axes of the tumors were measured using digital Vernier calipers. Tumor volumes were calculated using the following formula: (longest tumor diameter) × (shortest tumor diameter)² × 0.5. HCT116 xenograft mouse models were established by s.c. inoculation of 2 × 10⁶ HCT116 cells in Matrigel (BD Biosciences) and has been previously described (23). The tumors were allowed to grow until they reached ∼50 to 100 mm³ (day 8), at which point the first group received placebo (PBS) and the second group received chemotherapy [5-FU (15 mg/kg, days 8–12 and 15–19) and oxaliplatin (2 mg/kg, days 8 and 15)], and each treatment group contained eight animals. The tumors were measured three times a week in two dimensions using a caliper. The statistical significance was analyzed using the unpaired two-tailed Student’s t test.

TGF-α/VEGF ELISA

An equal number of cells were plated into 24-well plates and incubated for 24 h. Cells were treated in each experiment for a particular period. Conditioned medium or serum was collected in vitro and in vivo, respectively, and TGF-α or VEGF levels were analyzed according to the ELISA kit instructions (Calbiochem).

ADAM-17 activity

Excised tumors from HCT116 xenografts were homogenized in radioimmunoprecipitation assay buffer using an IKA Labortechnik homogenizer. After centrifugation of tissue homogenates, the supernatants were transferred to a new tube and protein concentration was determined. The InnoZyme TACE Activity kit (Calbiochem) was used to measure ADAM-17 (TACE) activity in 500 μg of each protein sample. Pure ADAM-17 was used as a positive control (+TACE).

Phospho-RTK array

Activity of a panel of RTKs was detected using an antibody-based array from R&D Systems. Antibodies against 42 different RTKs were prespotted in duplicate on nitrocellulose membranes, and cell lysates from EV control cells and ADAM-17–overexpressing cells were incubated with the membrane. Thereafter, a pan–anti-phosphotyrosine antibody was used to detect the phosphorylated tyrosine on activated RTKs.

Statistical analysis

Two-way ANOVA test was used to determine the significance of change in levels of apoptosis between different treatment groups. All changes in levels of apoptosis that are described as significant had P values that were <0.05 (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The nature of the interaction between GW280264X and chemotherapy was determined by calculating the combination index (CI) according to the method of Chou and Talalay (24).

Results

Chemotherapy treatment results in an acute increase in HER ligand shedding in CRC cells and xenografts

In view of our previous data showing that cytotoxic chemotherapy activates an EGFR-mediated survival response in CRC cells (5), we examined whether 5-FU treatment induced the release of the prototypical EGFR ligand TGF-α in culture medium of CRC cells. 5-FU–based regimens [FOLFOX: 5-FU/leucovorin + oxaliplatin; FOLFIRI: 5-FU/leucovorin + irinotecan (CPT-11)] represent the cornerstone of treatment of patients with advanced CRC. Twenty-four hours following treatment with 5-FU, we

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found a dose-dependent increase in TGF-α ligand shedding in HCT116 cells (Fig. 1A, top). In addition, shedding of other HER ligands, such as the EGFR ligand AREG [soluble AREG (sAREG)] and the HER3 ligand heregulin [soluble heregulin (sHeregulin)], also increased significantly following treatment with 5-FU in HCT116 cell line (Fig. 1A, bottom). Importantly, these effects were also observed in vivo, with statistically significant acute increases in human TGF-α shedding into the circulation observed following treatment of HCT116 CRC xenograft-bearing mice with 5-FU for 24 hours (Fig. 1B, top). In addition, the serum levels of AREG and heregulin were also increased in the 5-FU treatment group compared with control (Fig. 1B, bottom). The relevance of HER ligand shedding for drug resistance was shown by cotreating HCT116 cells with recombinant TGF-α, AREG, EGF, or heregulin and 5-FU. Treatment with each HER ligand significantly protected cells from 5-FU–induced apoptosis and apoptosis induced by oxaliplatin and the active metabolite of irinotecan, SN-38 (Fig. 1C, left and right; Supplementary Fig. S1). Taken together, these data suggest that induction of TGF-α, AREG, and heregulin ligand shedding is an acute mechanism of drug resistance in CRC cells.

Chemotherapy treatment results in an acute increase in ADAM-17 activity in CRC cells and xenografts

One mechanism by which EGFR can be activated is via ADAM-mediated ligand shedding. Treatment with 5-FU had no effect on TGF-α mRNA or AREG mRNA expression and resulted only in a moderate ∼2-fold increase in heregulin mRNA expression level (Supplementary Fig. S2). These results indicate that increased shedding of TGF-α, AREG, and heregulin following 5-FU treatment is predominantly due to posttranslational mechanisms. With this in mind, we investigated whether a number of different ADAM family members play a significant role in regulating chemotherapy-induced EGFR activation and TGF-α shedding by using gene-specific siRNAs directed against ADAM-9, ADAM-10, ADAM-12, ADAM-15, and ADAM-17 (Fig. 2A and B). Using quantitative real-time PCR, we found a decrease of ∼70% to 80% in ADAM-9, ADAM-10, ADAM-12, ADAM-15, and ADAM-17 mRNA expression following transfection with each siRNA in the LoVo and HCT116 cell lines (Supplementary Fig. S3). Specific downregulation of ADAM-17 protein was also confirmed by Western blotting (Fig. 2A). Interestingly, we found that the increased TGF-α shedding and EGFR activity following
5-FU treatment was only abrogated by ADAM-17 gene silencing, whereas silencing of the other ADAMs (ADAM-9, ADAM-10, ADAM-12, and ADAM-15) did not significantly affect 5-FU–induced TGF-α shedding and EGFR activation (Fig. 2A, top, and B). We next directly investigated the effect of chemotherapy treatment on ADAM-17 activity. We found that treatment with 5-FU significantly increased ADAM-17 activity in HCT116 cells (Fig. 2C). Importantly, this was also observed in vivo, with a significant increase in ADAM-17 activity observed in HCT116 xenografts exposed to 75 mg/kg 5-FU for 24 h (Fig. 2D). These results correlated strikingly with the effect of chemotherapy treatment on HER ligand shedding in vitro and in vivo (Fig. 1).

To rule out the possibility that the effects of ADAM-17 were specific to the Kras mutant and p53 wild-type HCT116 and LoVo models, we extended these studies into three further CRC cell line models: a p53-null HCT116 daughter cell line, the Braf-mutant and p53 wild-type RKO cell line, and the Kras wild-type/Braf wild-type and p53-mutant H630 cell line. We found that silencing of ADAM-17 attenuated 5-FU–induced EGFR activation in all three of these cell lines, indicating that these effects are not p53, Kras, or Braf dependent (Fig. 3A).

Importantly, we found that shedding of TGF-α following 5-FU treatment was ADAM-17 dependent in HCT116, LoVo, and H630 cell lines (Fig. 3B). In addition, 5-FU–induced AREG and heregulin shedding was completely abrogated when ADAM-17 was silenced in HCT116, LoVo, and H630 cell lines (Fig. 3C). We also determined the role of ADAM-17 in regulating an EGFR-mediated survival response following SN-38 and oxaliplatin (Supplementary Fig. S4). We found that silencing of ADAM-17 inhibited SN-38–induced TGF-α shedding and EGFR activation in HCT116, LoVo, RKO, and H630 cell line using ADAM-17 siRNA (Fig. 3B). In addition, 5-FU–induced AREG and heregulin shedding was completely abrogated when ADAM-17 was silenced in HCT116, LoVo, and H630 cell lines (Fig. 3C).

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with our earlier studies (5), oxaliplatin failed to increase EGFR activity in HCT116 cell line, and no significant increase in TGF-α shedding following oxaliplatin was observed in this cell line.

Taken together, these results indicate that CRC cells respond to chemotherapy by increasing ADAM-17 activity, which further regulates HER ligand shedding and activation of EGFR.

**Inhibition of ADAM-17 activity, using siRNA or the pharmacologic inhibitor GW280264X, sensitizes CRC cells to chemotherapy treatment**

We next investigated the effect of ADAM-17 inhibition on chemotherapy-induced apoptosis in the CRC cell lines models using ADAM-17 gene silencing. A significant supra-additive/synergistic increase in apoptosis was observed in ADAM-17–silenced CRC cells cotreated with 5-FU (Fig. 4A). Similar results were obtained when ADAM-17 siRNA was combined with SN-38 or oxaliplatin in this cell line panel (Supplementary Fig. S5A).

We next assessed the effect of pharmacologic inhibition of ADAM-17 using a small-molecule dual ADAM-10/ADAM-17 inhibitor, GW280264X, and compared this with a specific ADAM-10 inhibitor, GI254023X. Physiologically relevant doses that have been used in previous publications were used (25). We found that GW280264X inhibited 5-FU–induced ADAM-17 activity, TGF-α ligand shedding, and EGFR activity in both HCT116 and LoVo cell lines.
cells (Fig. 4B and C). In contrast, the ADAM-10–specific inhibitor GI254023X had only a marginal effect on 5-FU–induced TGF-α shedding and EGFR activation (Supplementary Fig. S6). In addition, when GW280264X was combined with 5-FU, SN-38, or oxaliplatin in HCT116 and LoVo cells, significant supra-additive/synergistic increases in apoptosis were observed (Fig. 4D; Supplementary Fig. S5B and C). In contrast, GI254023X had no effect on chemotherapy-induced apoptosis (Fig. 4D; Supplementary Fig. S5B and C). We next determined the level of synergy between GW280264X and chemotherapy using the method of Chou and Talalay to calculate CI values. For the combination of GW280264X with 5-FU and SN-38, CI values <0.7 were observed for majority of concentrations, indicative for strong synergism in HCT116 and LoVo cells (Supplementary Fig. S7A and B). The combination of GW280264X with oxaliplatin was also synergistic for the majority of drug concentrations, with most CI values between 0.5 and 1.0 (Supplementary Fig. S7A and B). These results further highlight the importance of ADAM-17 as a key mediator of resistance to chemotherapy and strongly suggest that ADAM-17–targeted agents may be novel drugs for use in conjunction with existing chemotherapy regimens in patients with CRC.

**Effect of ADAM-17 overexpression on growth/survival of CRC cells and xenografts**

To complement our gene silencing and inhibitor studies, we developed HCT116 cell line models that stably overexpress HA-tagged ADAM-17. Two stable clones with different levels of ADAM-17 overexpression were identified: HA-ADAM-17 3 (AD3) and HA-ADAM-17 4 (AD4; Fig. 5A). ADAM-17 activity in clones AD3 and AD4 were approximately 2.5- and 35-fold higher compared with EV

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**Fig. 4.** ADAM-17 mediates apoptosis following chemotherapy treatment in CRC cells. A, determination of apoptosis in CRC cells treated with combinations of ADAM-17 siRNA with chemotherapy. CRC cells were transfected with 10 nmol/L scramble control or 10 nmol/L ADAM-17 siRNA for 4 h and thereafter treated with 2.5 μmol/L 5-FU (HCT116 and HCT116-p53null), 1 μmol/L 5-FU (LoVo and RKO), and 0.5 μmol/L 5-FU (H630) cells for 72 h. The cell cycle status of the cells was monitored by flow cytometry after PI staining. Percentages of cells in sub-G1 phase are given. Representative results of at least three independent experiments are shown. B, HCT116 and LoVo cells were incubated with increasing concentrations of GW280264X for 4 h and thereafter treated with 5 μmol/L 5-FU for 24 h. Top, ADAM-17 activity was measured using an ADAM-17 fluorometric assay; bottom, TGF-α ligand shedding in cell culture medium was assessed by ELISA. C, HCT116 and LoVo cells were incubated with increasing concentrations of GW280264X for 4 h and thereafter treated with 5 μmol/L 5-FU for 24 h. Phospho-EGFR and EGFR expression was detected by Western blotting. Equal loading was assessed by probing for β-tubulin. D, determination of apoptosis in CRC cells treated with combinations of GI254023X or GW280264X and chemotherapy. HCT116 and LoVo CRC cells were pretreated with 2.5 μmol/L GI254023X or 2.5 μmol/L GW280264X and combined with 2.5 μmol/L 5-FU for 72 h. The cell cycle status of the cells was monitored by flow cytometry after PI staining. Percentages of cells in sub-G1 phase are given. Representative results of at least three independent experiments are shown. ***, P < 0.001.
Importantly, TGF-α, AREG, and heregulin shedding (Fig. 5A, left and right, bottom) and EGFR and HER3 activation (Fig. 5A) in the ADAM-17-overexpressing clones were significantly increased compared with the EV cell line, with the more highly ADAM-17-overexpressing clone (AD4) exhibiting greater TGF-α shedding and EGFR activation. In vitro, ADAM-17 overexpression protected cells from 5-FU-induced apoptosis (Fig. 5B; Supplementary Fig. S8B) and also apoptosis induced by oxaliplatin and SN-38 (Supplementary Fig. S8A and B).

We next determined the effect of increased expression of ADAM-17 on the growth of human HCT116 xenografts and evaluated their response to combined treatment with 5-FU and oxaliplatin (Fig. 5C). The growth of ADAM-17-overexpressing CRC xenografts was more rapid than control xenografts. Importantly, overexpression of active ADAM-17 in vivo attenuated the antitumor activity of 5-FU/oxaliplatin combination treatment (Fig. 5C). Western blot analysis confirmed that the ADAM-17-overexpressing xenografts expressed HA-tagged ADAM-17 (Fig. 5D, top). Furthermore, ADAM-17 activity levels were significantly higher (~4-fold) in ADAM-17-overexpressing tumors, and this was associated with increased EGFR phosphorylation in these tumors compared with the EV xenografts.
Consistent with our previous findings, we found that ADAM-17 activity levels increased significantly in EV controls and ADAM-17–overexpressing HCT116 xenografts following treatment with 5-FU and oxaliplatin (Fig. 5D, bottom). Moreover, chemotherapy-induced ADAM-17 activity levels were associated with increased EGFR phosphorylation (Fig. 5D, top). In addition, chemotherapy-induced caspase-3 activation was abrogated in the ADAM-17–overexpressing CRC xenografts compared with EV xenografts. Collectively, these data indicate that ADAM-17 activity regulates the sensitivity of CRC tumors to standard chemotherapy treatment, further indicating that combining ADAM-17 inhibitors with chemotherapy could be a potential novel strategy for the treatment of CRC.

Active ADAM-17 activates several growth factor RTKs, such as IGF-IR and VEGFR

To further investigate the mechanisms of ADAM-17–mediated resistance to chemotherapy treatment, we assessed the phosphorylation status of 42 RTKs in HCT116 cells, 24 hours following transient transfection with ADAM-17, using a human phospho-RTK array kit (Fig. 6A). In addition to EGFR, we found increased phosphorylation levels of VEGFR2/3, IGF-IR, PDGFR, the Ephrin receptors and developmental tyrosine kinase in ADAM-17–overexpressing HCT116 cells. In contrast, phosphorylation levels of hepatocyte growth factor receptor were reduced following transient transfection with ADAM-17 (Fig. 6A; Supplementary Fig. S9). The VEGFRs, PDGFR, and IGF-IR are key regulators of colorectal tumor angiogenesis, lymphangiogenesis, tumor growth, and proliferation and have emerged as important targets in CRC (26–28). So, we next validated our array results for these receptors by Western blotting analysis using phospho-specific VEGFR1, VEGFR2/3, IGF-IR, and PDGFR antibodies that reflect the activation state of the receptors. We found that VEGFR2/3, VEGFR1, and IGF-IR activity was significantly increased in the stable ADAM-17–overexpressing clones.

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**Fig. 6.** ADAM-17 regulates activity of several survival receptors such as VEGFR1, VEGFR2/3, and IGF-IR. A, human phospho-RTK array in HCT116 cells. HCT116 cells were transiently transfected with EV or HA-ADAM-17 expression construct for 24 h followed by protein extraction. The cell extracts were incubated with membranes containing antibodies to 42 different RTKs. The membranes were washed and incubated with a cocktail of biotinylated detection antibodies to measure the levels of active kinases. The RTKs that phosphorylation changed following 24 h of transfection with ADAM-17 construct are illustrated. B, Western blot analysis of HA, phospho-VEGFR2/3, phospho-VEGFR1, phospho-IGF-IR, and phospho-PDGFRβ expression levels in pcDNA 3.1 (EV control), HA-ADS, and HA-AD4 clones. Equal loading was assessed by probing for β-tubulin. C, expression levels of soluble IGF-I (sIGF-I) and soluble VEGF (sVEGF) in EV control, HA-ADS, and HA-AD4 clones were assessed by Western blotting and ELISA, respectively. D, HCT116 cells were incubated with 2.5 μmol/L GW280264X (GW) for 4 h and thereafter treated with 5 μmol/L 5-FU for 24 h. Soluble IGF-I and soluble VEGF ligand shedding in cell culture medium was assessed by Western blotting (left) and ELISA (right). ***, P < 0.001.
cell lines AD3 and AD4 compared with the EV cell line (Fig. 6B). Activity of PDGFRβ was only slightly increased in AD clones compared with the EV cell line. Moreover, we found that shedding of the VEGF and IGF-I ligands was significantly increased in cells stably overexpressing ADAM-17 (Fig. 6C). We subsequently assessed whether VEGF and IGF-I shedding occurred following chemotherapy treatment and if this was also regulated by ADAM-17. We found that 5-FU treatment resulted in increased VEGF and IGF-I ligand shedding and that this was abrogated in the presence of the ADAM-10/ADAM-17 inhibitor GW280264X (Fig. 6D). Taken together, these results indicate that through its ability to shed multiple growth factors, ADAM-17 is an important regulator of several key survival pathways following treatment of CRC cells with chemotherapy.

Discussion

We previously reported that CRC cells respond acutely to chemotherapy by activating a HER-mediated survival response and are thereby sensitized to HER inhibitors (5). In light of this, we investigated the mechanisms by which HERs are activated in response to chemotherapy in CRC cells. Initially, we assessed shedding of TGF-α and other HER ligands following exposure to 5-FU treatment both in vitro and in vivo. We found that 5-FU treatment resulted in statistically significant increases in human TGF-α, AREG, and heregulin shedding both in culture medium of HCT116 cells and in serum of mice bearing human HCT116 xenografts. Furthermore, addition of exogenous EGFR ligands to the culture medium resulted in decreased 5-FU–induced cell death, showing the functional role of TGF-α ligand shedding following chemotherapy treatment. Hagan et al. (2) showed that radiation therapy can increase shedding of TGF-α in serum of patients treated for hormone-refractory prostate cancer. Our study is the first to show increased HER ligand shedding in the context of chemotherapy treatment in CRC.

Several reports have indicated that different ADAMs, such as ADAM-9, ADAM-10, ADAM-12, ADAM-15, and ADAM-17, can induce EGFR activation by cleaving the extracellular domain of six ligands of EGFR (13), resulting in their shedding and ability to activate the receptor in an autocrine and paracrine manner. Hence, we determined the effect of ADAM-9, ADAM-10, ADAM-12, ADAM-15, and ADAM-17 gene silencing on TGF-α shedding and EGFR activity following 5-FU treatment. In both HCT116 and LoVo cell lines, complete inhibition of 5-FU–induced TGF-α shedding and EGFR activation was only observed following ADAM-17 silencing. More importantly, we found that ADAM-17 activity was potently upregulated following 5-FU treatment in CRC cells and that chemotherapy (the clinically relevant 5-FU and oxaliplatin combination) significantly increased ADAM-17 activity in human HCT116 xenograft models. These results correlated with the results of the TGF-α ELISA both in vitro and in vivo. Moreover, we found that ADAM-17 regulated 5-FU–induced, SN-38–induced, and oxaliplatin-induced HER ligand shedding and EGFR activation in a broad panel of CRC cell lines, irrespective of p53, Kras, or Braf mutational status. Furthermore, a synergistic activation of apoptosis was observed in vitro when ADAM-17 siRNA or the small-molecule ADAM-10/ADAM-17 inhibitor GW280264X was combined with chemotherapy treatment in this panel of CRC cells. In contrast, the specific ADAM-10 inhibitor GI254023X had no effect on chemotherapy-induced apoptosis. Taken together, our findings suggest that chemotherapy treatment results in acute upregulation in ADAM-17 activity, which promotes EGFR ligand shedding and an EGFR-mediated prosurvival response following chemotherapy treatment. Thus, targeting ADAM-17 in combination with chemotherapy could represent an important treatment strategy for patients with metastatic CRC.

To complement our gene silencing and small-molecule inhibitor studies, we further examined the importance of ADAM-17 activity as a mediator of resistance to chemotherapy treatment using ADAM-17–overexpressing HCT116 cell lines. ADAM-17–overexpressing clones showed increased ADAM-17 activity; TGF-α, AREG, and heregulin ligand shedding; and EGFR/HER3 activation. Moreover, we showed for the first time that ADAM-17 can also regulate shedding of other growth factors, such as IGF-I and VEGF, and subsequently regulates activity of their respective receptors, IGF-IR and VEGFR. Of note, the enhanced tumor growth of AD4 PBS xenografted mice compared with EV PBS xenografted mice may be the result of ADAM-17 regulating growth factor shedding and activity of multiple survival receptors that promote xenografts growth. It may be that ADAM-17 should be added to a growing list of nononcogenes that could be exploited as an anticancer drug target (29). Importantly, the clones with increased ADAM-17 activity levels had a decreased response to chemotherapy treatment compared with the EV clones. Moreover, overexpression of ADAM-17 protected HCT116 xenografts from the growth-inhibitory effects of chemotherapy and abrogated chemotherapy-induced apoptosis in vivo. Collectively, these results further indicate that ADAM-17 is an important regulator of chemotherapy resistance, and suggest that targeting this ADAM in conjunction with chemotherapy may have therapeutic potential for the treatment of CRC tumors.

Although a number of studies have shown additive interactions when chemotherapy was combined with the ADAM-10/ADAM-17 inhibitor INC3619 in lung cancer, breast cancer, and head and neck xenograft models (6, 30), no underlying mechanism behind this interaction was provided. Our data show for the first time that chemotherapy treatment can result in potent increases in ADAM-17 activity in CRC cells and that this protease thereby regulates resistance to chemotherapy treatment. Blocking this survival response, using small-molecule ADAM-10/ADAM-17 inhibitors, resulted in synergistic increase in apoptosis, and this was irrespective of p53, Kras, or Braf mutational status. One other study has investigated the role of...
ADAM-17 in CRC and only examined the interaction between an ADAM-17 inhibitor and EGFR-targeted agents (31); the current study is the first to examine the interaction between ADAM-17 and cytotoxic chemotherapy in CRC. We have previously shown that the death receptor ligand tumor necrosis factor–related apoptosis-inducing ligand can increase TGF-α shedding and HER1/HER2 activity and that this may be regulated by ADAM-17 in CRC, indicating that activation of ADAM-17 may be a common prosurvival response following treatment with a range of cytotoxic and apoptosis-inducing agents (32). Merchant et al. showed that ADAM-17 is overexpressed in primary and metastatic CRC compared with normal colonic epithelium, further highlighting the importance of ADAM-17 as a potential target in CRC (31).

In conclusion, our findings provide strong evidence that CRC tumors respond to chemotherapy by activating ADAM-17, which results in increased growth factor shedding and activation of growth factor receptor–mediated prosurvival response. Furthermore, we provide strong evidence that enhanced ADAM-17 activity and HER ligand shedding result in resistance to chemotherapy treatment in CRC tumors. Moreover, therapies targeting ADAM-17 (and thereby the activity of multiple RTKs, such as EGFR, HER3, IGF-IR, and VEGFR) in conjunction with existing chemotherapy treatments (FOLFOX and FOLFIRI) may enhance response rates in patients with advanced CRC and thereby improve survival rates compared with those obtained with combined EGFR monoclonal antibody inhibition (cetuximab)/chemotherapy treatment (33).

Disclosure of Potential Conflicts of Interest

P.G. Johnston: shareholdings, Fusion Antibodies and GlaxoSmithKline; consultancy, AstraZeneca, Pfizer, Roche Pharmaceuticals, Merck, Amgen, Bristol-Myers Squibb, and Ortho Biotech; contracted research, AstraZeneca and Amgen. The other authors disclosed no potential conflicts of interest.

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